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THE ACTION OF PROTEOLYTIC ENZYMES ON THE ANTITOXINS AND PROTEINS IN IMMUNE SERA.

II.—HEAT DENATURATION AFTER PARTIAL ENZYME ACTION.

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IN the first paper of this series (Pope, 1939) results were given dealing with the true digestion of the proteins present in immune horse-sera. It was pointed out there that when the proteolytic enzyme used was pepsin, the initial digestion-rate was not maintained when the conditions were adjusted to conserve a reasonable amount of the antitoxic activity. The use of more acid conditions or higher temperatures led to increased protein digestion, but was accompanied by extensive destruction of the antitoxin. Many experiments were made in an attempt to find conditions which would allow a satisfactory protein digestion without such a loss of antitoxin and some of these are reported here. At the time they were made the significance of the results obtained was not fully appreciated, and it was only later, after other experiments on heat denaturation of proteins had been done, that the two sets of experimental results were correlated.

EXPERIMENTAL.

The Effect of Heating at Low pH Values Before Enzyme Action.

In some of the experiments an attempt was made to alter the proteins present by heating in acid solutions at varying temperatures. It was hoped that partial denaturation of the protein would make it more easily digested by the enzymes at pH values of the order of 4·0. Some of the experiments are briefly recorded here.

Antitoxic plasma, diluted with two volumes of water, was made 0·5 per cent. with sodium citrate and adjusted to pH 4·4 with solid citric acid. This solution was heated to 58° C. for one hour; all the protein separated from solution as a semi-solid gel, and the filtrate from a portion appeared free

from all but traces of protein or antitoxin. Pepsin (0·5 per cent.) was added to the cooled gel mixture, which was then re-heated to 50° C. for 7 hours to determine the extent to which this denaturated protein was digested. After 7 hours little solid protein remained; tests on the filtrate showed that it contained 15·3 per cent. of its original protein in heat-coagulable form, and 61·5 per cent. of the original antitoxin. The protein and antitoxin determinations were made as previously described (Pope, 1939).

Similar material was heated at pH 5·2 and 58° C. for 4·5 hours. When cooled, it was adjusted to pH 4·2 and 1 per cent. pepsin added. After 24 hours' digestion at 37° C. the filtrate contained 86 per cent. of the original antitoxin.

In another experiment antitoxic pseudoglobulin was heated at pH 4·2 for 3 hours at 60° C. All the protein separated in insoluble form and the filtrate contained no antitoxin. After cooling to 37° C. 1 per cent. pepsin was added, and the temperature kept at 37° C. for 24 hours. Tests then showed that more than 50 per cent. of the original antitoxin was present in the filtrate.

The effect of " pre-heat " treatment at varying pH values was investigated in more detail on an antitoxic pseudoglobulin solution. The dilute protein solution was adjusted to varying pH values with citric acid only (no additional citrate), and then heated for 1 hour at 58° C. After cooling, all were adjusted to pH 4·2 and 0·05 per cent. pepsin added. After 12 hours at room temperature the mixtures were heated to 37° C. Owing to the lower electrolyte content protein precipitation was not quite as complete as in the experiments reported earlier. Table I shows the initial pH values, the change in pH during 1 hour at 58° C., and the antitoxin which remained in the filtrates after heating but before pepsin treatment. After adding pepsin, results are given for 12 hours at room temperature and for 6 hours and 30 hours at 37° C. It will be seen that the antitoxin liberated by peptic treatment depended on the pH at which the " pre-heat " treatment was carried out.

TABLE I.—*Percentage Amount of Antitoxin in Solution at Varying Points Before and After Pepsin Treatment* (*pH* 4·2 ; *Temperature* 37° *C.*).

Sample.	pH.		Before pepsin treatment. Per cent. antitoxin in solution after heating.	After pepsin treatment. Per cent. antitoxin in solution.		
	Initial.	After 1 hour at 58° C.		Room temperature overnight.	6 hours (37° C.).	30 hours (37° C.).
A	3·01	3·23	0	10·5	10·5	5
B	3·32	3·58	0	24	34	34
C	3·49	3·75	5	34·5	45	45
D	3·79	4·03	10	45·5	61·5	61
E	3·96	4·22	24	65·5	74·5	73
F	4·32	4·53	29	74·5	83·5	81

A more detailed examination was made after these solutions had been digested with pepsin for 48 hours at 37° C. (Table II). The increased purity

varied from 1·58 to 4·5 times, the amounts both of protein and of antitoxin which reappeared in the filtrates being dependent on the pH at which the "pre-heat" treatment was carried out.

TABLE II.—*Results after 48 Hours' Peptic Treatment of Antitoxin Solution Pre-heated at Varying pH Values.*

Sample.	pH at which "pre-heated".	Per cent. soluble antitoxin.	Per cent. soluble protein.	Units/g. protein.	R value (increased purity).
A	3·01	..	..	..	..
B	3·32	34	7·5	46,000	4·5
C	3·49	45	14·9	30,900	3·0
D	3·79	61	25·0	24,800	2·44
E	3·96	72	40·0	18,300	1·8
F	4·32	81	51·4	16,000	1·58

From these experiments it will be seen that antitoxin showed a peculiar behaviour after heating under certain acid conditions. Although such treatment rendered the protein, and with it the antitoxin, insoluble (particularly when the electrolyte content was high), it was found that pepsin and other proteolytic enzymes had the property of acting on such insoluble material, and that some of the protein and antitoxin became soluble again. Other examples of this method of treatment have already been given (Pope, 1938).

A number of other reagents such as alcohol, acetone and urea precipitate proteins, but it was found that pseudoglobulin solutions rendered insoluble by means of these reagents did not yield soluble antitoxin when treated with enzymes. There is, therefore, a marked difference between antitoxic protein which has been made insoluble by the special heat treatment described, and that made insoluble by alcohol or similar reagents. An explanation of the differences has already been given (Pope, 1938).

As a result of these and other observations, it was decided to make further study of the relationship between the denaturation of protein and the loss of antitoxic activity under varied conditions of acidity and temperature. By chance a starting material was selected which later was found to possess special characteristics. In the routine ammonium sulphate concentration process a precipitate is separated at about 30 per cent. saturation, which consists of fibrinogen, euglobulin, and contains some antitoxin. The antitoxin present can be recovered by solution of the precipitate, followed by reprecipitation with ammonium sulphate. The product so obtained is relatively impure, but was available in fairly large amounts and was used for experimental purposes. Some of the experiments carried out with this type of antitoxin solution are described here.

The Effect of Heating after Enzyme Action.

Antitoxin solution recovered from the first ammonium sulphate precipitate was diluted to contain 1·6 per cent. protein, and 5 per cent. sodium chloride added as electrolyte. Samples were adjusted to various pH values and heated at different temperatures. Insoluble protein, where formed, was removed by

filtration, and the residual protein and antitoxin in the filtrates determined. From the results (Tables III and IV) it will be seen that there was clear differentiation between the amount of protein rendered insoluble and the amount of

TABLE III.—*Effect of Heating at pH 4·2 and Varying Temperatures.*

Temp.	Time (min.).	Soluble protein (per cent.).	Soluble antitoxin (per cent.).	Units antitoxin/g. protein.	R value.
55° C.	15	57·1	82·5	10,600	1·44
	30	42·3	65·5	11,400	1·55
	45	32·1	58·5	13,400	1·82
	60	22·1	48·0	16,100	2·17
	120	10·7	31·0	21,300	2·90
58° C.	15	67·3	84·5	9,200	1·25
	30	29·0	67·0	17,000	2·31
	45	15·9	58·5	27,000	3·68
	60	12·65	51·5	30,000	4·07
	120	7·4	36·0	36,000	4·85
60° C.	5	72·5	93·0	9,100	1·23
	10	53·4	84·5	11,600	1·58
	15	26·6	70·5	19,500	2·65
	20	17·0	59·5	25,000	3·5
	25	15·7	59·5	27,200	3·79
62° C.	5	75·5	93·0	9,050	1·23
	10	45·0	79·5	12,900	1·76
	15	25·0	67·0	19,700	2·68
	25	9·7	49·0	37,000	5·05

TABLE IV.—*Effect of Heating.*

pH.	Temp. (° C.).	Time. (min.).	Soluble protein (per cent.).	Soluble antitoxin (per cent.).	Units/g. protein.	R value.
4·4	62	10	48·2	79·5	12,000	1·65
		15	26·8	67·0	18,400	2·50
		20	17·8	58·5	24,200	3·29
		25	11·4	48·5	31,900	4·25
		40	8·34	39·5	34,900	4·75
	64	10	39·4	76	14,100	1·93
		15	20·0	60·5	22,000	3·02
		20	13·6	53·5	28,700	3·94
		25	12·4	50·0	29,600	4·03
		40	8·07	41·5	37,500	5·13
4·6	58	25	66·4	89·5	9,600	1·35
		45	50·6	82·5	11,600	1·63
		60	43·1	77·0	12,750	1·78
		90	35·5	68·5	13,700	1·93
		120	29·2	65·0	16,250	2·22

antitoxic activity lost, and that the effect produced could be influenced by the pH, temperature, and time of heating. It was surprising to find that antitoxin could survive such drastic treatment, but additional experiments with similar antitoxic material confirmed these results.

Different results were obtained when this treatment was applied to antitoxic pseudoglobulin isolated between 30 and 50 per cent. saturation with ammonium sulphate. The protein present was rapidly rendered insoluble on heating in acid solution, and the amount of protein and antitoxin left in solution at any stage was, within experimental error, the same.

This difference in behaviour was traced ultimately to the action of fibrinolysin on the pseudoglobulin. This enzyme is associated with the fibrinogen in the precipitate produced at 30 per cent. saturation with ammonium sulphate, and its action appears to be enhanced by the tricresol added as an antiseptic to the plasma. The enzyme was isolated in impure form from fibrin clots produced by the addition of calcium chloride to oxalated plasma. After the addition of calcium, the plasma was shaken to obtain a sponge-like clot, and the fibrin so obtained washed with water, extracted with dilute acetic acid, and used in that form for experiments with the 30–50 per cent. ammonium sulphate fraction of antitoxic pseudoglobulin. While no definite evidence could be found that the enzyme converted any of the protein into a non-coagulable form, it did alter its properties, so that critical heat denaturation allowed the removal of a large amount of protein for little antitoxic loss.

After obtaining these results with fibrinolysin the effect of pepsin, trypsin and papain was investigated. The results using pepsin will be given in some detail, and the special conditions which are required for the other enzymes mentioned later.

Antitoxic pseudoglobulin obtained between 30 and 50 per cent. saturation with ammonium sulphate was diluted to about 1·5 per cent. protein, 5 per cent. sodium chloride added and the pH adjusted to 4·2. The solution was divided into two portions, 0·25 per cent. pepsin added to one, and both kept at room temperature for 24 hours. Heating was carried out for periods up to 30 minutes

TABLE V.—*Changes in Protein and Antitoxin in a Pseudoglobulin Solution on Heating to* 58°–59° *C. at pH* 4·2 *Before and After Pepsin Treatment.*

	Time (min.).	Protein left (per cent.).	Antitoxin left (per cent.).	R value (increased purity).
Without pepsin	5	80·4	92·5	1·15
	10	55·2	69·5	1·25
	15	21·1	21·2	1·00
	20	14·9	16·4	1·10
	30	5·95	6·5	1·09
With pepsin	5	74	92·5	1·25
	10	58	86·5	1·50
	15	45	81·0	1·80
	20	38·4	76·0	1·96
	30	32	72·5	2·32

at 58°–59° C. Table V shows that before enzyme action the protein and antitoxin became insoluble together, whereas after the enzyme had altered the protein there was definite separation.

When sodium chloride was used as the electrolyte, the protein precipitate formed on heating was rather gelatinous and filtration was slow. The substitution of ammonium sulphate for sodium chloride gave better filtration without affecting the results. Using the same antitoxic material described for the last experiment, a dilution was prepared of the same protein content ; the dilute material contained 15 per cent. ammonium sulphate and was adjusted to pH 4·2. The results after 30 minutes' heating at 58° C. are shown in Table VI.

TABLE VI.—*Effect of Substituting Ammonium Sulphate for Sodium Chloride as the Precipitating Electrolyte. (Heated to 58° C. at pH 4·2 for 30 min.)*

Pepsin (per cent.).	Protein left (per cent.).	Antitoxin left (per cent.).	Units/g. protein.	R value.
0	20	20	12,100	1·0
0·25	37	83	26,800	2·24

Some idea of the stability of antitoxin on heating at high temperatures in acid solution after slight modification of the protein by enzyme action is given in the next experiment, which was carried out on antitoxic pseudoglobulin of high titre. Antitoxic pseudoglobulin solution containing about 5000 units per ml. was diluted to 2 per cent. protein and 15 per cent. ammonium sulphate added. The pH was adjusted to 4·2 and 0·25 per cent. pepsin added. The solution was heated for a total period of 2 hours at 60° C. and the results given in Table VII obtained.

TABLE VII.—*Results after Heating Pepsin-treated High Titre Antitoxic Pseudoglobulin at 60° C. and pH 4·2.*

Time (min.).	Protein left (per cent.).	Antitoxin left (per cent.).	Units/g. protein.	R value.
30	40	76	55,700	1·9
60	35·8	75·5	62,500	2·11
120	33·6	74	65,200	2·20

An important question is that of the amount of pepsin required to produce the change in the protein molecule which confers this stability to heat on antitoxin. Antitoxic pseudoglobulin, diluted to about 1·5 per cent. protein and containing 5 per cent. sodium chloride, was adjusted to pH 4·2. Varying amounts of pepsin were added, and after standing for 30 minutes at room temperature, the solutions were heated for 30 minutes at 58° C. Table VIII shows that the change can be produced by quite small amounts of pepsin. The differences in R value for the varying amounts of pepsin used were almost within the limits of experimental error, when it is considered that each sample was heated separately.

TABLE VIII.—*Action of Varying Amounts of Pepsin.* (*Solutions Heated* 30 *min. at* 58° *C.*)

Pepsin (mg./100 ml.).	Protein left (per cent.).	Antitoxin left (per cent.).	Units/g. protein.	R value.
50	35·7	74	24,700	2·08
100	39·5	74	22,300	1·87
150	36·1	79	26,200	2·19
200	41·2	81	23,500	1·97
250	40·2	81	24,100	2·00

As the change in the pseudoglobulin molecule could be produced by a small amount of pepsin, the question of the amount of true protein digestion which took place in the conversion of the antitoxic globulin from the non-heat-stable to heat-stable condition was investigated. The same antitoxic globulin concentrate used in the last experiment was diluted as before, adjusted to pH 4·2 and 0·125 per cent. pepsin added. Measurement of the change in total protein content at room temperature showed that little digestion was taking place, and the observations were therefore made at 40° C. Table IX shows that, up to 270 minutes, the loss of heat-coagulable protein amounted to only about 10 per cent. At the end of 30 minutes at 40° C. the protein lost by digestion was 4 per cent., and tests made with rabbit anti-albumin serum showed that the digested protein was almost certainly albumin present as a contamination in the pseudoglobulin solution. In an earlier experiment it was shown that 30 minutes' treatment with pepsin at room temperature was sufficient to confer heat stability at 58° C. and pH 4·2. Even less true digestion could have taken place under these conditions than at 40° C.

TABLE IX.—*Extent to which Protein in Pseudoglobulin Solution was Digested* (*Pepsin* 0·125 *per cent., pH* 4·2, *temp.* 40° *C.*).

Time (min.).	Original protein undigested (per cent.).
30	96·5
60	98
90	97·5
120	95
150	96
180	93·5
210	92
240	92·5
270	90

An additional observation of the rate at which pepsin acts on the antitoxic protein is given in the next experiment. The same antitoxic globulin solution was diluted to contain about 1·5 per cent. protein and 10 per cent. sodium chloride and adjusted to pH 4·2. Pepsin (0·1 per cent.) was added, and the

solution heated to 60° C. as rapidly as possible and samples taken at intervals. The results (Table X) show that without allowing any time for pepsin action before heat treatment, 10 minutes' heating at 60° C. increased the antitoxin purity by 2·15 times for a loss of 12·5 per cent. of the antitoxin.

TABLE X.—*Results Obtained when the Solutions were Heated to 60° C. as rapidly as possible after adding Pepsin.*

Time at 60° C. (min.),	Soluble antitoxin in filtrate per cent. of original.	Units/g. protein.	R value.
10	87·5	20,600	2·15
15	79·5	23,200	2·42
20	76·0	24,100	2·51
30	71·5	24,700	2·58
40	70·0	26,800	2·80
50	65·5	27,300	2·85
60	62·5	27,800	2·90

All the experimental work on heat denaturation so far recorded was done on a small experimental scale, and further work was carried out using moderately large volumes to determine the practical possibilities of the method. When working with large volumes it was impossible to secure such rapid heating of the solutions from room temperature to about 60° C. without the use of special apparatus. The experiment was done using a large water-bath, thermostatically controlled, and a stainless steel bucket. The heat transfer through the bucket was good, and rapid stirring was maintained until the required temperature was reached. At the commencement of the heating the water-bath temperature was 10° C. higher than required, and was finally adjusted when the contents of the bucket reached correct temperature. 2·5 litres of concentrated antitoxic globulin solution were diluted to 14 litres and adjusted

TABLE XI.—*Data for the Concentration of a 20-litre Batch of Antitoxic Pseudoglobulin Previously Concentrated by Ammonium Sulphate.*

Stage at which sample taken.	Protein left (per cent.).	Antitoxin left (per cent.).	Units/g. protein.	R value.
Control dilution	100	100	13,000	1·00
Prior to heating	..	97	..	..
When temperature reached (° C.):				
40	60	93	20,200	1·55
45	58	93	21,000	1·61
50	52	83·5	21,200	1·63
55	33	72·0	28,900	2·22
At 55° C. for (hours):				
1	27	72·0	35,400	2·72
2	25·5	72·0	36,800	2·82
3	24·6	72·0	38,000	2·92

to pH 4·2 with citric acid. Pepsin (20 g. = 0·1 per cent. of final volume) was added. At this stage the experiment was unavoidably delayed for 48 hours. Saturated ammonium sulphate solution (6·0 litres) was added, and the solution heated to 55° C. as described and kept at that temperature for 3 hours. Samples were taken at various stages and the results obtained on them are recorded in Table XI. In this experiment the protein had been reduced to 24·6 per cent. of that originally present for a loss of 30 per cent. (approximately) of the antitoxin and the antitoxin purity increased by nearly 3 times.

Far the greater part of the work recorded was carried out on antitoxic pseudoglobulin fractionated from serum or plasma by means of ammonium sulphate. It was felt that the absence of other non-antitoxic proteins, such as fibrin, euglobulin and albumin, would make it more easy to study the changes which were taking place. This idea was confirmed when an attempt was made to apply the data acquired from work with pseudoglobulin to the treatment of plasma. It was found that at about pH 4 the albumin digested very slowly, and in the presence of albumin the required alteration in the pseudoglobulin molecule was incomplete in a reasonable time at room temperature. Attempts to increase the digestion by use of higher temperatures were not too successful ; any over-digestion of the pseudoglobulin led to a failure in the heat denaturation stage of the process and resulted in reduced purity of the final product. Eventually these difficulties were overcome by pepsin treatment at a very low pH for a short period at room temperature. The albumin was rapidly converted to a non-heat-coagulable stage and allowed the required change to take place in the pseudoglobulin. Any further enzyme action was then stopped by readjustment of the pH, the addition of a high concentration of ammonium sulphate, and rapid heating to high temperature to denature and precipitate the non-antitoxic protein. The successful treatment of either whole blood or plasma requires also a consideration of the action of antiseptics which assist in the denaturation process, and of methods for the removal of lipoids from the final product.

Results from other experiments where other enzymes were used may now be considered ; the action of fibrinolysin is shown in the next experiment. To 100 ml. of antitoxic pseudoglobulin was added 100 ml. of autolysed fibrin solution and 800 ml. water. After adding 10 per cent. sodium chloride the solution was adjusted to pH 4·2 and incubated for 24 hours at 39° C. Small samples were heated rapidly to 60° C. After 15 minutes at 60° C. the filtrate contained 65 per cent. of the antitoxin and showed the usual marked increase in purity. The antitoxin had also the characteristic property of rapid flocculation with toxin. A control experiment made without the addition of any fibrin autolysate showed no heat stability and the filtrate was almost antitoxin-free. The fibrin autolysate used was prepared by allowing fresh fibrin clots to autolyse at 37° C. in phosphate buffer pH 8 containing 0·2 per cent. tricresol. All the solid fibrin went into solution, but the protein could be removed by heat coagulation or by salting out with ammonium sulphate. Similar experiments were made using the acetic acid extracts from fibrin clots ; these extracts produced a change in the globulin molecule which allowed the application of the heat-denaturation method. No evidence could be found that the fibrinolysin produced any true digestion of the proteins.

The first attempts to use crude trypsin solutions met with difficulties. When small amounts of enzyme were used, digestion took place very slowly. Large amounts of enzyme produced rapid digestion, but caused the destruction of antitoxin at a proportional rate. The difficulties were overcome in two ways. Antitoxic pseudoglobulin solution, diluted to contain 1·5 per cent. protein, was adjusted to pH 4·3, heated to 60° C. for 10 minutes and then cooled. After adjusting to pH 8, 5 per cent. of Cole's pancreatic extract solution (Cole, 1916) was added and the solution allowed to stand at 18° C. for 24 hours. Portions of the solution then received 10 per cent. sodium chloride, were adjusted to pH 4·2 and heated at 60° C. After 20 minutes at 60° C. the filtrate contained 65 per cent. of the antitoxin and the purity had increased by 2·76 times. In this method of treatment advantage was taken of the fact that heating in acid solution caused denaturation changes in the pseudoglobulin molecule which affected the non-antitoxic portion. This portion was then available for attack by a low concentration of trypsin, while the antitoxic portion, which was not denatured, was unaffected by the enzyme.

A second method which is available for trypsin depends on the fact that whereas this enzyme does not produce normal digestion of protein in acid solutions, it does cause the pseudoglobulin molecule to divide into two protein fragments under these conditions.

10 ml. of concentrated antitoxic globulin (high titre) was diluted to 100 ml., 10 per cent. sodium chloride added and adjusted to pH 4·2. Trypsin solution (5 ml.) was added and the solution incubated at 37° C. for 24 hours. The filtrate at this stage contained 85 per cent. of the antitoxin, although a very heavy precipitate had formed. After heating for 15 minutes at 60° C. the filtrate contained 76 per cent. of the antitoxin and the increased purity was 2·2 times. The material showed properties similar to those obtained by pepsin treatment (see Table VII).

The experiments described in this paper led to a successful routine method of producing serum of increased purity. Antiseptic assists in producing the desired protein denaturation which occurs on heating. The original plasma containing 0·4 per cent. of tricresol is diluted with two volumes of water before adjustment of the pH and addition of pepsin.

Lipoids, if not removed, may later cause clouding of the serum. It was found that the addition of toluol to the mixture before heating caused the lipoid to come out with the coagulated protein when heating to 55° C. for two hours was employed.

True digestion of the proteins to a non-coagulable state makes it impossible to use differential heat coagulation for their separation; moreover, true digestion would cause the production of proteose-like substances, which would make very difficult the salting-out of the purified antitoxin. True digestion does not occur when the enzyme action is limited. A disaggregation, and not true digestion, is therefore arranged to take place under conditions suitable for the enzyme chosen, *e. g.* for pepsin a pH of 3·2, a temperature of 20°–23° C. and a period of 30 minutes.

The enzyme action is then stopped by adjusting the pH to 4·1 with sodium hydroxide and adding a high concentration of an electrolyte to precipitate some protein and the enzyme; the whole mixture is then heated rapidly to the

coagulation temperature of the non-antitoxic protein. The filtrate is adjusted to pH 7, and the antitoxin is precipitated by making the ammonium sulphate concentration 30–32 per cent. After filtration the precipitate is pressed and dialysed.

At a final protein content of 16 per cent. the product shows an increased value in units per ml. of 8 to 12 times; the yield of antitoxin should be at least 60 per cent. in the final product. Optical examination reveals only a trace of Tyndall beam, showing that no denatured protein remains in pseudo-solution in the product.

DISCUSSION.

The experimental work reported in this paper may be divided into two portions: that in which the antitoxin was first treated so that partial denaturation occurred before enzyme treatment was applied; and the reverse of this method, where, after a slight amount of enzyme action, the proteins are separated by critical heat denaturation. Of these two methods, the second gives the more complete separation of non-antitoxic protein.

All the evidence which has been obtained in this work supports the view already put forward (Pope, 1938) that in its normal form, as it exists in horse-serum, the antitoxic globulin consists of a complex molecule which is easily disaggregated by certain enzymes into two portions. Of these, one is non-antitoxic and is readily denatured in acid solutions; the other portion carries the antitoxic activity and, fortunately, is far less readily denatured. This second portion is, however, readily denatured by agents such as alcohol, acetone or urea, and if either the original molecule or the separated antitoxic portion is denatured by these reagents, subsequent treatment with enzymes does not regenerate antitoxic activity. Similarly, excessive heat treatment (such as boiling the original antitoxic solution or the separated antitoxic portion) or heating in solution at too low a pH causes a permanent loss of antitoxic activity.

The experiments reported in this paper have shown that the disaggregation of the molecule is produced by very small amounts of enzyme, in very short time, and that no measurable amount of pseudoglobulin is digested to a stage where it ceases to be coagulable on boiling at the isoelectric point. Once this minor change has been produced, it is advantageous to stop further enzyme action by the addition of a high concentration of electrolyte, and to heat as rapidly as possible to a temperature at which denaturation of the non-antitoxic portion takes place.

A point of some interest arises from the results obtained with trypsin. When added in low concentration to antitoxic globulin solutions at pH 8 the enzyme acts but slowly, and many weeks' treatment at 37° C. are required to obtain a measurable increase in the antitoxin purity as a result of protein digestion. Large amounts of trypsin cause the formation of a precipitate, more rapid and extensive digestion, but such loss of antitoxic activity that no increased purity results. An observation of a similar kind to those reported for antitoxic sera was made in connection with the digestion of beef muscle by trypsin for the production of culture medium. It is customary to heat the

muscle to 80°–90° C. to destroy the so-called antitryptic properties, otherwise little digestion takes place on adding trypsin. If, however, the muscle is adjusted to pH 4·0 for a while and later adjusted back to pH 8, digestion by trypsin proceeds as though the muscle had been heated to 80°–90° C. The acid treatment, by producing a denaturation change in the protein, allows the trypsin to attack the substrate readily.

On the basis of the observations recorded, routine methods have been worked out for either whole blood or plasma as a starting material. As was expected, it was found necessary to make many adjustments to the methods recorded in this paper before a technique suitable for routine use was found. For example, the presence of proteins other than pseudoglobulin caused some trouble, and led either to incomplete disaggregation of the antitoxin molecule, or too extensive hydrolysis when the treatment with enzyme was more drastic. The details of a routine process based on the disaggregation of the antitoxic protein followed by critical heat denaturation are given in this paper.

SUMMARY.

It has been shown that the antitoxic globulin protein can be partially denatured by heat treatment in acid solution, with the result that the antitoxin becomes insoluble. By treatment with proteolytic enzymes the undenatured antitoxic portion of the globulin molecule may be split off and regained in soluble form. Other methods of denaturation may lead to total loss of antitoxic activity, since they denature both portions of the molecule.

The reverse of this process is that of a primary slight action of proteolytic enzymes on the antitoxic globulin molecule which produces disaggregation into two protein fragments. Of these, the non-antitoxic portion is removed by critical heat denaturation.

The second method gives the greater increase in purity of the antitoxin. Results are given for pepsin, trypsin and fibrinolysin.

Details of a method for the purification and concentration of antitoxic sera are given. After very limited enzyme action on the antitoxic protein, the non-antitoxic protein is removed from solution by critical heat denaturation. The antitoxin remaining in solution is then salted out with ammonium sulphate.

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THE PREPARATION OF DIPHTHERIA ANTITOXIN IN A STATE OF HIGH PURITY.

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In previous papers (Pope, 1938, 1939*a*, *b*) the disaggregation of the antitoxic protein molecule by limited enzyme action followed by removal of non-antitoxic protein by means of critical heat denaturation has been reported. Starting with plasma of high antitoxic titre, it has been found possible to obtain an end-product having 86,000 units of antitoxin per g. of protein. So far this value has not been exceeded by the use of the methods already reported. The degree of purity which can be achieved by this process is limited because of certain factors which are discussed here.

This method, when applied to normal horse-serum, removes 95 per cent. or more of the proteins which were present originally. The small amount of protein which survives the treatment is either a portion of the pseudoglobulin molecule, or is due to some undetected antibodies present in the normal serum used. Applied to antitoxic sera, further limits are imposed, due to changes in the horse proteins as a result of immunization, and in particular (in the case of diphtheria antitoxic sera) to the fact that antibodies other than true antitoxin have been produced, *e. g.* anti-bacillary protein antibodies.

Some attempts have been made to determine a value for antitoxin in terms of units per g. of protein, by taking advantage of the specific precipitation between antitoxin and toxin. Ramon (1923) dissociated the specific precipitate by means of heat and acid, and estimated about 60,000 antitoxic units per g. of protein. Locke and Main (1926) used a similar procedure, and obtained a similar order of value. Marrack and Smith (1930), working on the nitrogen content of floccules, deduced a rather higher value of about 90,000 units/g. protein. More recently Pappenheimer and Robinson (1937) have given a figure of 0·0016 mg. nitrogen per antitoxic unit. This was obtained from determination of the nitrogen content of floccules, after deducting the nitrogen due to the toxin which was present. This would give a value of the order of 100,000 units per g. of protein.

The observation was made independently by both Pappenheimer and Robinson (1937) and ourselves that the floccules produced by the interaction of enzyme modified antitoxin and toxin contained less nitrogen than those produced from ordinary antitoxin. From a large number of observations we have obtained an average value of 140,000 units per g. of protein for such altered antitoxin.

As a result of a large amount of work on the nitrogen content of floccules,

we have considerable doubt as to the true value for the unit/g. protein value for ordinary antitoxin. Some of the reasons for these doubts may be considered here.

It is generally assumed that after their interaction, toxin and antitoxin produce a complex which is completely separated from solution. On this assumption the total number of toxin and antitoxin units used are taken into consideration in deducing the number of antitoxic units per g. of protein. That such an assumption is unwarranted can be shown either by an optical examination (Pope and Healey, 1938) of the supernatant liquid after centrifuging off the floccules (such solutions show a dense Tyndall beam owing to incompletely separated complex), or by finding how much additional antitoxin can combine with the complex in the supernatant to form TA_2 (Healey and Pinfield, 1935). In an exceptional case we found as much as 50 per cent. of the complex remained in the supernatant after prolonged centrifuging. These observations apply much more strongly to ordinary antitoxin than to enzyme treated antitoxin. With the latter material, flocculation is very rapid and complete. We have found it equally difficult to wash the floccules with saline without losses due to dispersed floccules which remain in the supernatant after centrifuging. Their presence in the washing liquid can be detected by the methods mentioned. Our observations suggest that up to 20 per cent. of the specific complex may be lost in these ways, with the result that the purity figure deduced from the determination becomes too high.

There is, however, another factor which needs consideration. Marrack and Smith (1930) stated that the floccules carried down very little pigment or similar material when they separated from solution. While this observation may have appeared to be true from small-scale work, large-scale operations with the floccules from 35 l. of toxin as reported later in this paper showed the presence of much pigment material and of lipoid. Any nitrogen present in these substances would tend to reduce the purity figure for the antitoxin.

The figures for the two kinds of antitoxin may be taken as of the order of 60–90,000 units for ordinary and 140,000 units for enzyme-treated antitoxin per g. of protein.

It occurred to us that it might be possible to isolate the enzyme-modified antitoxin in reasonable quantity by specific precipitation with toxin, washing the complex so formed, and then dissociating the product to free the antitoxin. As our attempts to repeat Ramon's (1923) method of dissociation with heat and acid had not given us satisfactory results, a new method was tried. By treating the washed complex with acid and a small amount of pepsin, the toxin in the complex was destroyed. Details of the action of pepsin on the complex will be reported later; here it is sufficient to say that 30 minutes' treatment with pepsin at pH 3·0 and at 18° C. resulted in the liberation of about 70 per cent. of the calculated antitoxin content of the floccules. After careful fractionation with ammonium sulphate to remove inert material the product was obtained in concentrated form. On this material, direct estimation of the antitoxic titre (flocculation method) and of protein (heat coagulation refractometric method) gave a figure of 135,000 units of antitoxin per g. of protein. All the protein present appeared to be specifically precipitable by diphtheria toxin.

The agreement between this figure of 135,000 for direct determination and the figure of 140,000 deduced from measurements of floccule nitrogen is very close. For our calculations we have used the same figure for toxin nitrogen as Pappenheimer and Robinson (0·00046 mg. nitrogen per Lf. unit), and deducted the nitrogen due to toxin from the total nitrogen found in the floccules.

The antitoxin obtained in this way was examined for reactions other than those of antitoxin. No evidence could be obtained of precipitation between bacillary protein materials from P.W. 8 strain of *C. diphtheriæ* and this antitoxin, nor did this antitoxic material cause agglutination of P.W. 8 suspensions. The peptic concentrate from which it was prepared reacted with bacillary protein and agglutinated P.W. 8 strain of *C. diphtheriæ*.

The chemical properties of this product are being investigated and will be reported later.

EXPERIMENTAL.

Diphtheria toxin containing 42 Lf. units per ml. was measured with aseptic precautions into 9 bottles, each receiving 3880 ml. To each bottle was added 25 ml. of enzyme-purified antitoxin containing 6500 units per ml. Flocculation occurred within 10 minutes at room temperature, and the floccules were allowed to settle overnight. Part of the supernatant liquid was drawn off, and the floccules then recovered by centrifuging. After washing, twice with phenol saline and once with distilled water, the floccules were re-suspended in distilled water to a volume of 2 l. The suspension was adjusted to pH 3·0 with solid citric acid and 0·5 g. of pepsin added. At pH 3 the floccules became redissolved, and the solution produced was intensely brown in colour. After 30 minutes at 18° C. tests showed nearly 70 per cent. of the calculated antitoxin had been set free. The solution was adjusted to pH 7. During neutralization a small precipitate appeared at pH 4·5. It was very deeply coloured, but could not be removed completely by filtration. The neutral solution was carefully treated with solid ammonium sulphate until a small but highly-coloured precipitate separated and the filtrate was water-clear. The antitoxin in the clear filtrate was separated by means of sulphate, pressed and dialysed. For a protein content of 9·6 per cent. the dialysed material contained 12,900 flocculation units of antitoxin per ml.; antitoxic units/g. protein, 135,000.

The figures for the experiment were :

Total antitoxic units used to prepare floccules . .	1,465,000
Total antitoxic units set free after pepsin . .	1,012,000

Yield, 69·5 per cent.

SUMMARY.

The purity of diphtheria antitoxin in terms of units of antitoxin per g. of protein has been considered. For ordinary antitoxin the figure would appear to be between 60–90,000 units/g. protein ; some of the difficulties that must be taken into account in deducing this value from floccule nitrogen determinations have been pointed out.

For antitoxin altered by enzyme treatment, the average figure obtained from floccule nitrogen determinations is 140,000 units/g. By isolation through specific toxin precipitation, and dissociation with pepsin, antitoxin has been obtained in highly purified form, and direct estimations of protein and antitoxin gave a value of 135,000 units/g. protein.

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THE INFLUENCE OF THE PANCREAS AND OF PANCREATIC PREPARATIONS USED FOR THE EXTRACTION OF CERTAIN FRACTIONS FROM *BACT. AERTRYCKE* ON THE OXYGEN UPTAKE OF BRAIN AND MUSCLE SUSPENSIONS.

A CORRECTION.

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IN a previous paper (Delafield and Smith, 1936) it was recorded that certain fractions derived from *Bact. aertrycke* had the property of increasing *in vitro* the initial oxygen uptake of brain suspensions in the presence of glucose, and of inhibiting the oxygen uptake of muscle suspensions in the presence of succinate. These fractions were obtained by a process of tryptic digestion of the bacterial bodies followed by alcohol precipitation. This property was possessed only by those bacterial fractions which were toxic, and produced marked blood-sugar changes when injected into rabbits. It was thought that these modifications of tissue respiration, demonstrated to occur *in vitro* under the influence of the toxic fractions, might possibly play some part in the mechanism of the toxic state in the living animal. Against this view, however, was the fact that the undigested dead bacterial bodies which are toxic did not have any significant effect on the oxygen uptake of the tissue suspensions.

Nevertheless it appeared to be possible that there was some constituent of the bacterial cell capable of modifying the oxygen uptake of tissue suspensions which was not dissolved out of the cell under the conditions of the experiment, but could be liberated by the process of tryptic digestion.

It has now been shown that the conclusions reached in our previous paper were entirely wrong in the sense that the modifications of tissue-cell respiration brought about by the fractions were due to some material which was present in the particular trypsin preparation first used in the digestion of the bacterial bodies, but was absent from the bacterial bodies themselves.

The work to be described establishes that a material which modifies the oxygen uptake of brain suspensions *in vitro* is :

(1) Present in certain trypsin preparations, but not in others.

(2) Absent from the *Bact. aertrycke* fractions when prepared by the use of trypsin preparations which have no effect on brain tissue respiration.

(3) Absent from *Bact. aertrycke* fractions prepared by a method not involving tryptic digestion.

(4) Present in the pancreas.

Further, it is established that the pancreas and all the trypsin preparations tested contain a material which inhibits the oxygen uptake of muscle in succinate.

EXPERIMENTAL.

METHODS.

The trypsin preparations.

1. The trypsin preparation originally used for the digestion of the bacterial bodies in the extraction of bacterial fractions which modified tissue respiration is a powder which will be called trypsin A. From it were prepared by autodigestion followed by precipitation with 68 per cent. alcohol, under conditions similar to those existing during the preparation of the bacterial fractions, white powders which will be referred to as trypsin crude AF68 and trypsin AF68.

(2) Other preparations of trypsin were used for comparison. Trypsin B is a liquid containing glycerol, trypsin C is a powder, and trypsin D is a powder which was described by the manufacturers as being " polysaccharide free ".

The bacterial fractions.

(1) Crude AF68 obtained from the dead bodies of *Bact. aertrycke* by digestion with trypsin A followed by fractionation with alcohol at a concentration of 68 per cent.

(2) AF68 obtained from crude AF68 by precipitating the calcium contained in the latter by the equivalent amount of oxalic acid, and following this by further alcohol precipitation.

(3) Crude DF68 made as in (1), but using trypsin D instead of trypsin A.

The tissue suspensions.

Brain and muscle suspensions from rabbits or from guinea-pigs were used. The brain was removed within a few minutes of the killing of the animal by a blow on the neck, ground up in a mortar without sand to a fine cream, suspended in Ringer solution and strained through muslin. Muscle from the back of a recently-killed animal was finely chopped and washed several times in a large volume of tap-water. After the water had been squeezed out the muscle was ground up in a mortar to a fine paste, and again suspended in tap-water. Some of this suspension was then poured on to muslin, and the retained muscle mass was rubbed with a watch-glass. The smaller particles passed through the fabric, and the tissue collected from the under-surface was in so fine a state of division that it could be pipetted accurately when re-suspended in tap-water.

Measurement of oxygen uptake.

This was determined in the Barcroft type of micro-respirometer. The Ringer solution used contained NaCl 9·0, $CaCl_2$ 0·24, and KCl 0·42 g. per litre, and when needed glucose was added to give a final concentration of 0·2 p.c. Succinate as substrate was used as a neutralized (pH 7·4) solution in a concentration of $M/6·5$. Phosphate buffer $M/15$ at pH 7·4 was always added to each cup. Carbon dioxide was absorbed by 20 per cent. potash placed in the central cup with a small roll of filter-paper.

The effect of a bacterial fraction or of a trypsin preparation or of a tissue extract was determined by comparing, in separate manometers, the oxygen uptake of the tissue suspension alone with its oxygen uptake when mixed with the material under test. The test material was added to both cups in order to balance any oxygen uptake that it might itself possess. Usually three or four manometers were used for each group, and the average of their readings taken, so that the error inherent in comparing single observations might be lessened.

The oxygen uptake is expressed in the tables as the $-Q_{O_2}$, *i. e.* the number of cubic millimetres of oxygen taken up in 1 hour by 1·0 mg. of dry weight of tissue. In a typical experiment the manometers were set up thus :

Group 1 (3 manometers).
- Left-hand cup : 1 c.c. buffer + 0·5 c.c. 1·2 per cent. glucose + 1·5 c.c. Ringer.
- Right-hand cup : 1 c.c. buffer + 0·5 c.c. 1·2 per cent. glucose + 0·5 c.c. Ringer + 1·0 c.c. brain suspension in Ringer.

Group 2 (3 manometers).
- Left-hand cup : 1 c.c. trypsin suspension in buffer + 0·5 c.c 1·2 per cent. glucose + 1·5 c.c. Ringer.
- Right-hand cup : 1 c.c. trypsin suspension in buffer + 0·5 c.c. 1·2 per cent. glucose + 0·5 c.c. Ringer + 1·0 c.c. brain suspension in Ringer.

RESULTS.

The effect of different untreated preparations of trypsin on the oxygen uptake of tissue suspensions.

Trypsin A, which was used in the manufacture of those *Bact. aertrycke* fractions which were found to modify the respiration of tissue suspensions, was found to have a pronounced effect on oxygen uptake. The oxygen uptake of brain in the presence of glucose was markedly increased, and that of muscle in the presence of succinate was greatly diminished. The three other trypsin preparations had little effect on the oxygen uptake of brain, although the respiration of muscle in succinate was diminished to a significant amount. In the absence of added glucose neither the trypsin preparations nor the bacterial fractions had any effect on respiration. Examples of experiments are recorded in Table I.

TABLE I.—*The Effect of Different Preparations of Trypsin on the Oxygen Uptake of Tissue Suspensions.*

The final concentration of the fractions was 1·0 mg. per c.c. except with the liquid trypsin B when the final concentration was 1 in 12.

		Substrate.	$-Q_{O_2}$.	Percentage effect on oxygen uptake.
1.	Brain alone	Glucose	2·1	
	,, + Aertrycke crude AF68	,,	3·4	+61
2.	,, alone	,,	2·8	
	,, + trypsin A	,,	5·9	+111
3.	,, alone	,,	2·1	
	,, + trypsin B	,,	1·8	−14
4.	,, alone	,,	2·4	
	,, + trypsin C	,,	2·6	+8
5.	,, alone	,,	2·4	
	,, + trypsin D	,,	2·5	+4
6.	Muscle alone	Succinate	4·1	
	,, + Aertrycke crude AF68	,,	1·4	−66
7.	,, alone	,,	4·8	
	,, + trypsin A	,,	0·7	−85
8.	,, alone	,,	5·1	
	,, + trypsin B	,,	2·5	−50
9.	,, alone	,,	4·8	
	,, + trypsin C	,,	3·2	−33
10.	,, alone	,,	3·4	
	,, + trypsin D	,,	2·2	−35

The effect of fractions obtained from trypsin A on the oxygen uptake of tissue suspensions.

Autodigestion of the trypsin A followed by alcohol precipitation yielded a white powder—trypsin crude AF68—which modified tissue respiration in the same way as the trypsin A, but not to the same extent. In one experiment

the trypsin A increased the oxygen uptake of brain by 111 per cent., whereas the trypsin crude AF68, acting on the same brain suspension, increased the uptake by 82 per cent.

The further treatment required to produce trypsin AF68 reduced the activity still further. This reduction of activity was also found in the bacterial fractions prepared with trypsin A. In one experiment with a brain suspension, Aertrycke crude AF68 increased the oxygen uptake by 62 per cent., Aertrycke AF 68 by 54 per cent., and a fraction which had been submitted to still further treatment with alcohol had only a 33 per cent. effect. On comparing the corresponding bacterial and trypsin fractions it was found that the bacterial fractions had a somewhat greater effect on both brain and muscle than the trypsin fractions. It was therefore a possibility that the bacterial bodies contained some substance which also modified tissue respiration, and so added to the activity which was shown to be possessed by trypsin A.

The effect of Bact. aertrycke *fractions prepared by means of trypsin D and also by the method of Boivin and Mesrobeanu on the oxygen uptake of tissue suspensions.*

Fractions prepared from the bodies of *Bact. aertrycke* using trypsin D instead of trypsin A were also found to be toxic to rabbits and to cause hyperglycæmia. The fraction prepared by the method of Boivin and Mesrobeanu (1933), which

TABLE II.—*The Effect of* Bact. aertrycke *Fractions Prepared by* (1) *Trypsin A*, (2) *Trypsin D, or* (3) *the Method of Boivin and Mesrobeanu, on the Oxygen Uptake of Tissue Suspensions.*

The final concentration of the fractions was 1·0 mg. per c.c.

	Substrate.	$-Q_{O_2}$.	Percentage effect on oxygen uptake.
1. Brain alone	Glucose	2·4	
,, + Aertrycke crude AF68 (trypsin A)	,,	4·3	+79
,, + ,, ,, DF68 (,, D)	,,	2·5	+4
2. ,, alone	,,	1·5	
,, + Aertrycke crude AF68 (trypsin A)	,,	2·4	+60
,, + ,, ,, DF68 (,, D)	,,	1·6	+7
3. Muscle alone	Succinate	3·5	
,, + Aertrycke crude AF68 (trypsin A)	,,	1·8	−49
,, + ,, ,, DF68 (,, D)	,,	3·0	−14
4. ,, alone	,,	2·0	
,, + Aertrycke crude AF68 (trypsin A)	,,	0·6	−70
,, + ,, ,, DF68 (,, D)	,,	2·0	±0
5. Brain alone	Glucose	2·4	
,, + Aertrycke crude AF68 (trypsin A)	,,	3·8	+58
,, + ,, fraction (Boivin)	,,	2·8	+17
6. ,, alone	,,	2·2	
,, + Aertrycke crude AF68 (trypsin A)	,,	4·4	+100
,, + ,, fraction (Boivin)	,,	2·4	+9
7. Muscle alone	Succinate	6·6	
,, + Aertrycke crude AF68 (trypsin A)	,,	1·4	−80
,, + ,, fraction (Boivin)	,,	5·7	−10
8. ,, alone	,,	4·9	
,, + Aertrycke crude AF68 (trypsin A)	,,	1·5	−70
,, + ,, fraction (Boivin)	,,	4·6	−6

consists in coagulating the bacterial protein with trichloracetic acid and, after spinning, precipitating with alcohol or acetone, was also toxic and produced hyperglycæmia. These bacterial fractions when examined were found to have no significant effect on the respiration of brain or muscle tissue suspensions in the presence of glucose or succinate respectively. It is noteworthy that although trypsin D had a significant influence on the oxygen uptake of muscle in succinate, the bacterial fraction prepared by its aid was almost inactive in this respect. Some of the results of experiments are set out in Table II.

From the results of the foregoing experiments it is clear that the effects attributed to some constituent of the bacterial cell were brought about by some material in the particular trypsin preparation used in digestion. This material might have been introduced into trypsin A during manufacture, or might have been present in the pancreas before manufacture. The absence from the other tryptic preparations of the power to modify tissue respiration might have been due to the absence of this active material from the pancreas used or to its destruction during manufacture.

The influence of fresh pancreatic extracts on these tissue respirations was accordingly examined.

The effect of pancreatic tissue suspensions on the oxygen uptake of brain and of muscle suspensions.

The pancreas was dissected out from a recently killed rabbit, freed of fat as far as possible, minced with scissors, and ground up in a mortar with the addition of $M/15$ phosphate buffer, pH 7·4. This suspension was then strained through muslin to remove coarse particles. Suspensions so prepared could be readily pipetted, and were found to contain amounts of dry weight of pancreas which ranged from 1·0 mg. to about 10·0 mg. per c.c., and had, when tested alone in 1·0 c.c. quantities, a negligible oxygen uptake. But when added to a brain tissue suspension the oxygen uptake of the latter was, in the majority of instances, significantly influenced when glucose was added as substrate. Thirty-one experiments were performed, but the results were not uniform. In five of these the pancreas had no significant effect; in four there was an actual diminution in the oxygen uptake of the brain suspension mixed with pancreas amounting to an average of 64 per cent. In the remaining 22 experiments there was an average increase in the oxygen uptake of 71 per cent., the range being from 14 to 122 per cent. Taking the 31 experiments together the mean $-Q_{O_2}$ of brain suspensions alone was 2·2, with a standard error of $\pm 0{\cdot}092$. The mean $-Q_{O_2}$ of the brain suspensions with added pancreas was 3·2, with a standard error of $\pm 0{\cdot}251$, and the difference between the two means was $1{\cdot}0 \pm 0{\cdot}267$. Pancreas suspensions therefore had a definitely significant effect in increasing the oxygen uptake of brain suspensions in this series of experiments, although in four experiments a diminution of the $-Q_{O_2}$ was demonstrated. No explanation of this diminution in the oxygen uptake was found, and it can only be conjectured that it was due to intrinsic variations in the brain or pancreas of individual animals.

Some experiments were also made without the addition of glucose, and in these the pancreas had no effect on the oxygen uptake of the brain suspension.

Five experiments were performed on the oxygen uptake of washed rabbit muscle in the presence of succinate, and in every instance addition of a pancreatic suspension caused a diminution in the oxygen uptake, which ranged from 24 to 77 per cent., with an average of 46 per cent.

The effect of tissue suspensions other than those of the pancreas on the oxygen uptake of brain suspensions.

A number of suspensions from other organs were examined in the same way as those from the pancreas. Increase in oxygen uptake was found to occur with some of them, but the increase was not, in general, as great as that brought about by the addition of pancreas. In eight experiments with liver suspensions there was an average increase of 38 per cent., ranging from 10 to 78 per cent. The adrenal in four experiments increased the oxygen uptake by an average of 29 per cent., ranging from 10 to 39 per cent. The thyroid had a negligible effect in four experiments. In two experiments with kidney suspensions the increase was 14 and 20 per cent. In 17 experiments with a suspension made from ground-up mucosa of the small intestine there was an increase in every case, with an average of 30 per cent., and a range of 6 to 71 per cent.

The effect of fractions derived from pig pancreas on the oxygen uptake of the brain and muscle suspensions.

Attempts were then made to obtain from the pancreas a material more active than the untreated gland. Fresh pig pancreas was obtained from the slaughter-house, and after removal of most of the fat, was finely minced and stirred up for half an hour with about three times its weight of 0·5 per cent. hydrochloric acid. Sodium carbonate solution was then added to bring the pH to about 4·7, when the mixture filtered readily through filter-paper. This filtrate was neutralized and tested. It was found to increase the one-hour oxygen uptake of brain in glucose by 65 per cent., and to diminish the oxygen uptake of muscle in succinate by 85 per cent. In a further experiment the filtrate after neutralization was heated to 100° C. for two minutes, and after separation of a precipitate which came down was found to cause in 1 c.c. amounts a 42 per cent. increase in the $-Q_{O_2}$ of brain in glucose, and a 65 per cent. reduction in that of muscle in succinate. The precipitate suspended in distilled water in a concentration of 1·0 mg. per c.c. diminished both brain and muscle oxygen uptake in glucose and succinate respectively by only 19 and 9 per cent. respectively. This experiment was repeated with the modification that the HCl extract was neutralized after heating, and was then found to give a 58 per cent. increase of oxygen uptake with brain in glucose, and a 53 per cent. reduction with muscle in succinate. The precipitate, which separated on heating, was suspended in Ringer, and found to have no activity with brain, and but a slight effect on muscle respiration.

On the addition to the hydrochloric acid extract of alcohol to produce a final concentration of 50 per cent., a white precipitate formed. This was

collected and dried. When tested in two experiments it gave at a final concentration in the respirometer of 1·0 mg. per c.c. increases of 81 and 78 per cent. in the oxygen uptake of brain in glucose, and a reduction of 91 per cent. in the oxygen uptake of muscle in succinate. An active material was extracted from the fresh mince by shaking for three hours with about four times its volume of 25 per cent. alcohol. The mixture was cleared in a Laval centrifuge, and to the translucent liquid was added an equal volume of alcohol. A precipitate which on washing with alcohol and drying was a white powder, was found to increase the oxygen uptake of brain in glucose in two experiments by about 50 per cent., and to lessen muscle respiration in succinate by 83 per cent.

Results of experiments are recorded in Table III.

TABLE III.—*The Effect on the Oxygen Uptake of Brain in Glucose and of Muscle in Succinate of Materials Obtained from Fresh Pancreas.*

Experiment.	$-Q_{O_2}$.	Percentage effect on oxygen uptake.
1. Brain alone	2·3	
,, + 1·0 c.c. acid extract of pancreas	3·8	+65
2. Muscle alone	3·8	
,, + 1·0 c.c. acid extract of pancreas	0·6	−85
3. Brain alone	2·6	
,, + 1·0 c.c. heated acid extract of pancreas	3·8	+46
4. Brain alone	2·4	
,, + 1·0 mg. per c.c. of material extracted by alcohol	3·5	+46
5. Muscle alone	4·8	
,, + 1·0 mg. per c.c. of material extracted by alcohol	0·8	−83
6. Brain alone	2·8	
,, + 1·0 mg. per c.c. of alcohol precipitate from acid extract	5·1	+81
7. Muscle alone	2·1	
,, + 1·0 mg. per c.c. of alcohol precipitate from acid extract	0·2	−91

The effect of dried pancreas on the oxygen uptake of tissue suspensions.

Owing to the difficulty of keeping fresh material, experiments were made with dried pancreas. Fresh pig pancreas, with most of the adherent fat removed by rough dissection, was finely minced, and stirred for a few minutes with five times its volume of acetone. The acetone was filtered off, the solid well washed once with acetone, and then dried in a vacuum desiccator over sulphuric acid. The dried mass was brown in colour, and could be readily reduced to a fine powder. Three preparations of this kind were made from different samples of pancreas. All of these were found to be active, and remained active after keeping for some months. In a total of 56 experiments, in all of which the final concentration of the dried pancreas was 1·0 mg. per c.c., the $-Q_{O_2}$ of brain in glucose was increased in every instance. The increases

ranged from 23 to 113 per cent., with an average of 65 per cent. On only three occasions was the increase less than 40 per cent. The mean $-Q_{O_2}$ of the brain alone was 2·6, but with dried pancreas added it was 4·3. The difference of these means is 1·7, and the standard error of this difference is 0·171. The dried pancreas in the same final concentration diminished the oxygen uptake of muscle in succinate by an average of 48 per cent. in three experiments. In one trial with brain washings (a brain suspension centrifuged to remove suspended matter), the dried pancreas lessened the oxygen uptake in the presence of succinate by 72 per cent.

From this dry pancreas an active material was extracted with dilute hydrochloric acid. The finely powdered acetone-dried pig pancreas was suspended in five times its volume of water, and to this suspension was added three times its volume of 0·5 per cent. HCl. The mixture was shaken for an hour, allowed to stand for another hour and then filtered. To the filtrate normal caustic soda was added till the pH was about 6·5. The liquid was then boiled. An inactive precipitate was filtered off. The filtrate was then concentrated by boiling under reduced pressure to about 30 c.c., cooled, and 150 c.c. of alcohol were added. A white precipitate was produced which was collected, washed with alcohol and ether and dried. In sixteen trials with brain in glucose it produced an increase in the $-Q_{O_2}$ on every occasion, which amounted to an average of 53 per cent., with a range between 12 and 128 per cent. This variability in the increase in the oxygen uptake was not explained. None of these alcohol precipitates had a definitely greater activity than the original dried pancreas from which they were derived.

The alcohol precipitate from the acid extract of dried pig pancreas is a white powder when dried which disperses in water to give an opalescent liquid. It contains intact protein, for in addition to positive biuret, tryptophane, Millon, ninhydrin and arginine reactions there is a precipitate on the addition of sulphosalicylic or of picric acid. The Molisch reaction is positive, but the reducing value by the Hagedorn-Jensen method is the equivalent of 7·7 per cent. of glucose. After heating for two hours at 100° C. in a normal concentration of HCl the reducing value increases, but only to an amount represented by 10 per cent. of glucose. Heating in normal acid for two hours, however, largely destroys the power of the material to increase the oxygen uptake of brain. In one experiment the $-Q_{O_2}$ increase due to the addition of 1·0 mg. per c.c. of the material was 128 per cent., but after hydrolysis with acid the increase was found, when tested on the same brain suspension, to have been reduced to 28 per cent.

Addition of acetone to the hydrochloric acid extract also resulted in the precipitation of a white material, which was found to be active on each of the four occasions on which it was tested, and gave an average increase of 72 per cent. in the $-Q_{O_2}$ of brain suspensions in glucose. Although this material was about 50 per cent. more active than the dried pig pancreas with which it was compared in these four experiments, it gave no better results than did the alcohol precipitate when tested on two occasions with the same brain suspension. Summarized results are recorded in Table IV.

The effect on the oxygen uptake of brain of material derived from a tryptic digest of dried pig pancreas.

Attempts were made to obtain a more active material from pancreas by removing proteins. Twenty grammes of dried pancreas were shaken with 100 c.c. of $N/2$ trichloracetic acid. The mixture was spun, and alcohol to a concentration of about 60 per cent. added to the supernatant. A white precipitate formed, which was then dried with alcohol and ether. This material was inactive. Tryptic digestion was next used to remove the proteins. Ten grammes of acetone-dried pig pancreas were ground up with 0·1 g. of a trypsin powder which had been shown to have no effect on the oxygen uptake of brain. The powder was suspended in 2000 c.c. of water, the reaction adjusted to pH 8·5 with decinormal soda, 100 c.c. of toluene added, and the liquid incubated for three days. The reaction was adjusted to pH 8·5 during the course of digestion by the addition of caustic soda on three occasions, and a further amount of trypsin powder was added after 36 hours. The mixture was then heated under reduced pressure to remove toluene, cleared by passing it three times through a Laval centrifuge, and then concentrated to 150 c.c. by heating under reduced pressure. To 50 c.c. of liquid 300 c.c. of alcohol were added. After standing overnight, a white precipitate was collected and dried. The yield from 50 c.c. was 0·52 g. This material was found to be active in increasing the $-Q_{O_2}$ of brain in glucose in six experiments in which the average increase was 68 per cent., ranging from 24 to 100 per cent. It was not, however, more active than the dried pig pancreas which was used as a comparison in the series of trials. Summarized results are recorded in Table IV.

TABLE IV.—*The Effect of Dried Pig Pancreas and of Materials Derived from it on the Oxygen Uptake of Brain Suspensions in Glucose.*

The figures for the $-Q_{O_2}$ are the average results of the number of experiments shown. The final concentration of the materials tested was 1·0 mg. per c.c.

	Number of experiments.		$-Q_{O_2}$.	Percentage effect on oxygen uptake.
1.	56	Brain alone	2·6	
		,, + dried pancreas	4·3	+65
2.	13	,, alone	2·9	
		,, + dried pancreas	4·9	+69
		,, + alcohol precipitate from HCl extract	4·5	+55
3.	4	,, alone	2·5	
		,, + dried pancreas	3·7	+48
		,, + acetone precipitate from HCl extract	4·3	+72
4.	6	,, alone	2·9	
		,, + dried pancreas	5·2	+79
		,, + alcohol precipitate from tryptic digest	4·8	+65

CONCLUSIONS.

The results obtained show that the modifications in the oxygen uptake of certain tissue suspensions previously described as being brought about by some constituent of the cells of *Bact. aertrycke* and set free by tryptic digestion are caused by some material present in the particular brand of trypsin which was originally used. This active material, being precipitable by concentrations of alcohol of 50 per cent. and upwards, was evidently present as a contaminant in the bacterial fractions which had been examined for their effect on the oxygen uptake of tissue suspensions. Similar bacterial fractions made by using other brands of trypsin or by a method not involving tryptic digestion of the bacterial bodies were found to have little if any effect on the oxygen uptake of tissue suspensions.

It was then found that an extract made from fresh pancreatic tissue by means of buffer solution had the effect of increasing the oxygen uptake of brain suspensions in glucose, and of inhibiting the respiration of muscle suspensions in succinate. In the majority of a number of trials this effect was demonstrated, but in a few experiments the extract was either inactive, or had an inhibiting effect on the oxygen uptake of brain suspensions. Whether these opposite results have any significance it is not possible to say, but it is conceivable that the state of secretory activity of the pancreas may bear some relation to these observations. The active material resists drying, for it was present in acetone-dried pancreas, and it could be extracted from both dried and fresh gland by means of buffer solution, dilute hydrochloric acid and dilute alcohol, but was precipitated as a white powder on increasing the concentration of alcohol to 50 per cent. The material gave positive tests for intact protein, but it has not been further examined chemically. When injected into rabbits it was not toxic, nor had it any effect on the blood-sugar of the animals. Whether these modifications of the oxygen uptake of tissue suspensions brought about *in vitro* by some substance in the pancreas are of physiological importance it is not at present possible to say.

SUMMARY.

1. The pancreas contains a material which greatly increases the oxygen uptake of brain suspensions in the presence of glucose, and diminishes the oxygen uptake of washed muscle suspensions in the presence of succinate.

2. A certain " trypsin " powder is active in the sense of bringing about both these modifications of oxygen uptake, but other " trypsin " preparations are inactive with brain suspensions, although they inhibit the oxygen uptake of muscle in succinate.

3. Fractions derived from *Bact. aertrycke* by a process involving tryptic digestion also bring about these modifications of oxygen uptake (as previously described), but only when prepared by means of a " trypsin " preparation, which itself influences the oxygen uptake of both brain and muscle suspensions.

4. Bacterial fractions prepared by means of " trypsin " preparations which have no effect on the oxygen uptake of brain suspensions in glucose, have no influence on the tissue respiration of either brain in glucose or of muscle in succinate.

Bacterial fractions prepared by methods not involving tryptic digestion are also inactive in this respect.

Our thanks are due to the Medical Research Council for a whole-time grant to one of us (H. A. S.), and for a grant in aid of expenses.

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A COMPARISON OF THE PHOTODYNAMIC ACTIVITY OF SOME CARCINOGENIC WITH NON-CARCINOGENIC COMPOUNDS.

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IN an earlier paper (Mottram and Doniach, 1938) we described the photodynamic activity of a group of carcinogenic agents. We showed that 1 : 2-benzanthracene and some of its derivatives sensitize living cells to near-ultra-violet radiation. This paper deals with an attempt to standardize this reaction, using cultures of *Paramœcium caudatum*. The reaction is compared with the effect upon paramœcia of some non-carcinogenic light-sensitizers.

MATERIAL AND METHODS.

Paramœcia.

Cultures 3 to 6 days old were used. These were subcultured in the dark in 5 per cent. Lemco broth in water.

Colloid.

Acetone solutions of 3 : 4-benzpyrene were added to water and the acetone pumped off. The hydrocarbon used was from a sample purified and stored in the dark to free it from photo-oxidation products. Fresh suspensions were used for each experiment. They fluoresce bright green in ultra-violet light through Wood's glass.

Light.

The source was a mercury-vapour lamp, voltage 103, current 3·4 amps. The light was filtered by a glass plate opaque to radiations below 3300 Ångström units.

Details of exposure.

Using the same pipette, 2 drops of colloid were added to 2 drops of paramœcium culture in the hollow of a well-slide. This was immediately placed on a lead block (for cooling purposes) 21 cm. from the centre of the lamp. By occasional rapid examination under a dissecting microscope the time taken to kill the paramœcia was measured. About half-way through the lethal time the movements of the organisms become sluggish. Then they commence a slow spin, and eventually they sink down motionless. After this they cytolyse whether left under the lamp or not. The end-point was determined when over 95 per cent. of the paramœcia lay motionless at the bottom of the hollow of the well-slide. Given any one culture and concentration of colloid the times taken to kill numerous samples of the mixture did not vary by more than 10 per cent. This time factor was used as an index of the photodynamic potency of the colloid used. The 2 drops of culture used in each reading were usually found to contain several hundred paramœcia. Paramœcia without colloid showed no effect even after some hours under the lamp. Paramœcia mixed with colloid and kept in the dark showed no lethal effect even after weeks of contact.

EXPERIMENTS.

Experiment 1.

Samples of a paramœcium culture were added to a series of dilutions of 3 : 4-benzpyrene suspension in water and immediately tested under the lamp. The time taken to kill each sample was determined. The opacity of suspensions stronger than 1 in 100,000 introduced a factor lengthening the lethal time by virtue of light absorption. The results are summarized in Table I and Fig. 1. In Fig. 1 lethal time is plotted against the logarithm of percentage concentration of the colloid. The curve brings out the extraordinary sensitivity of this reaction. Paramœcia were killed in less than 60 minutes in dilutions up

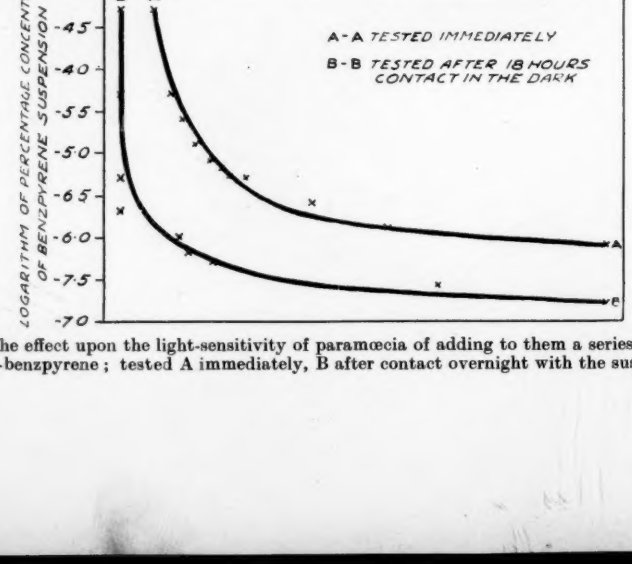

FIG. 1.—The effect upon the light-sensitivity of paramœcia of adding to them a series of dilutions of 3 : 4-benzpyrene ; tested A immediately, B after contact overnight with the suspension.

TABLE I.—*The Photodynamic Potency of a Group of Carcinogenic and Non-carcinogenic Light-sensitizers. The dilutions of the Compounds are given in the first column. The times taken by light to kill Paramœcia mixed*

TABLE I.—*The Photodynamic Potency of a Group of Carcinogenic and Non-carcinogenic Light-sensitizers. The dilutions of the Compounds are given in the first column. The times taken by light to kill Paramœcia mixed with the various colloids, both on immediate contact* (I) *and after contact overnight* (O), *are given in the other columns.*

Dilution of colloid in the mixture. 1 part in—	Exposure time in minutes taken to kill paramœcium-colloid mixtures under the lamp.															
	Eosin.		Quinine sulphate.		Acriflavine.		Acridine.		Methyl-cholanthrene.		Cholanthrene.		1 : 2-Benzanthracene.		3 : 4-Benzpyrene.	
	I.	O.	I.	O.	I.	O.	I.	O.	I.	O.	I.	O.	I.	O.	I.	O.
$2{\cdot}5\times10^3$	12	12	..	..	..	..	..	..	..	..	..	..	..	..	..	..
5×10^3	13	15	5	Toxic	10	Toxic	..	..	..	..	..	..	..	..	..	..
1×10^4	21	23	17	,,	19	,,	..	..	..	..	..	..	..	..	..	..
$1{\cdot}5\times10^4$	..	..	26	50	..	..	..	..	..	..	..	..	..	..	..	..
2×10^4	..	..	35	60	..	..	..	..	..	..	..	..	..	..	..	..
$2{\cdot}5\times10^4$	35	38	44	60	..	..	..	..	..	..	..	..	..	..	..	..
3×10^4	..	..	52	Over 60	..	..	..	..	..	..	..	..	..	..	..	..
5×10^4	55	59	..	..	36	Toxic	..	..	..	..	..	..	..	..	..	..
1×10^5	..	..	..	..	48	Over 60	..	..	..	..	..	..	..	..	..	..
2×10^5	..	..	..	..	60	..	4	3	11	6	7	$5\frac{1}{2}$	6	2	6	2
5×10^5	..	..	..	..	..	..	7	7	..	..	..	..	..	..	..	..
8×10^5	..	..	..	..	..	..	11	10	..	..	..	..	..	..	..	..
1×10^6	..	..	..	..	..	..	18	15	15	10	..	..	..	..	..	..
2×10^6	..	..	..	..	..	..	27	25	19	12	14	9	10	2	8	2
3×10^6	..	..	..	..	..	..	35	35	..	..	..	..	..	..	..	..
4×10^6	..	..	..	..	..	..	48	50	..	..	..	..	..	..	$9\frac{1}{2}$	2
5×10^6	..	..	..	..	..	..	55	Over 60	..	..	..	..	..	..	..	..
6×10^6	..	..	..	..	..	..	..	..	25	16	..	..	..	..	..	..
8×10^6	..	..	..	..	..	..	..	..	34	..	..	..	..	..	11	..
1×10^7	..	..	..	..	..	..	..	..	39	18	30	13	20	3	..	..
$1{\cdot}2\times10^7$	..	..	..	..	..	..	..	..	..	..	..	..	..	..	$12\frac{1}{2}$	..
$1{\cdot}6\times10^7$	..	..	..	..	..	..	..	..	..	..	..	..	..	..	14	..
$1{\cdot}8\times10^7$	..	..	..	..	..	..	..	..	..	..	..	..	..	..	15	..
2×10^7	..	..	..	..	..	..	..	..	..	25	45	22	25	6	17	2
4×10^7	..	..	..	..	..	..	..	..	..	..	..	..	..	..	25	..
5×10^7	..	..	..	..	..	..	..	..	60	36	54	30	40	18	..	2
8×10^7	..	..	..	..	..	..	..	..	..	..	..	..	..	..	34	..
1×10^8	..	..	..	..	..	..	..	..	..	60	60	54	55	28	..	9
$1{\cdot}2\times10^8$	..	..	..	..	..	..	..	..	..	..	..	..	..	..	60	..
$1{\cdot}5\times10^8$	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	10
2×10^8	..	..	..	..	..	..	..	..	..	..	..	60	..	36	..	13
4×10^8	..	..	..	..	..	..	..	..	..	..	..	..	..	55	..	40
6×10^8	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	60

to 1 in 100 millions. Even in dilutions of the order of 200 millions the paramœcia became sluggish within 1 hour, though they did not give a determinable end-point.

Experiment 2.

Contact in the dark with colloid was found to increase the sensitivity of paramœcia to light. A considerably shorter time-interval sufficed to kill them. Paramœcium culture was added to an equal volume of a 1 in 1 million suspension of 3 : 4-benzpyrene in the dark. At 5-minute intervals a sample of the mixture was tested under the lamp. The results are given in Fig. 2. It is seen that the lethal time for the mixture fell from 7 to 2 minutes in under half-an-hour's contact in the dark. After this, no increase in light-sensitivity was observed.

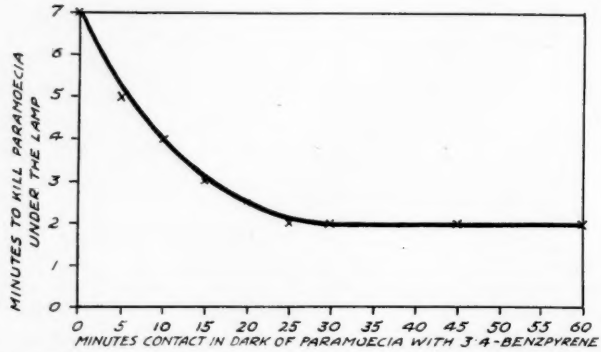

FIG. 2.—A 1 in 1 million suspension of 3 : 4-benzpyrene was added to an equal volume of paramœcium culture in the dark. At 5-minute intervals samples were withdrawn from the mixture and tested under the lamp. The time to kill the paramœcia is plotted against the time they had been in contact in the dark with the benzpyrene.

Experiment 3.

Experiments 1 and 2 above were combined. Paramœcia were added to a series of dilutions of 3 : 4-benzpyrene and tested after 18 hours' contact in the dark. The increase in sensitivity was marked. Paramœcia in dilutions of colloid up to 1 in 50 millions were all killed in 2 minutes. In greater dilutions the end-point was difficult to determine. Some organisms were killed rapidly; others appeared unaffected by the light. However, even in a dilution of 1 in 600 million the majority were killed in 60 minutes. The results are given in Fig. 1 and Table I.

Experiment 4.

A solution of water-soluble photo-oxidation derivatives of 3 : 4-benzpyrene was prepared as follows; A bottle containing water and a benzene solution of 3 : 4-benzpyrene was put in a sunny window for a few weeks. The colour of the upper (benzene) layer turned from a pale yellow to deep orange. The water became tinged with yellow. The watery part was separated, filtered,

and boiled down to a small volume. This solution was tested for photodynamic action upon paramœcia under standard conditions. When the lethal times for various dilutions were plotted graphically, a curve similar in slope to that given by 3 : 4-benzpyrene resulted (see Fig. 3). It could not be compared quantitatively, as one was ignorant of the exact concentration of oxidation-products present in the original solution. Contact in the dark with this solution

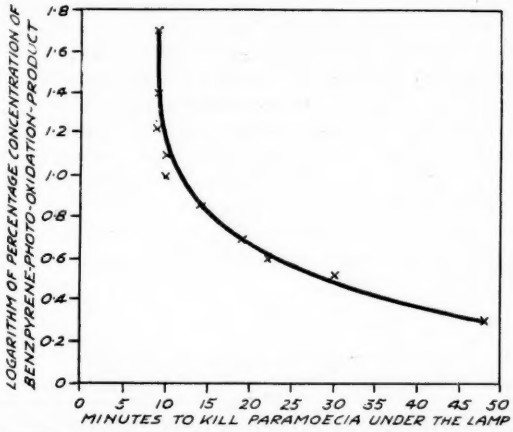

FIG. 3.—The effect upon the light-sensitivity of paramœcia of adding to them a series of dilutions of a watery extract of 3 : 4-benzpyrene in benzene irradiated by sunlight.

did not reduce the lethal time for paramœcia (see Table II). This interesting finding was confirmed numerous times. Thus, in this reaction to contact in the dark with paramœcia, we have a definite qualitative difference between the photodynamic action of 3 : 4-benzpyrene and that of its water-soluble photo-oxidation products.

TABLE II.—*The Time taken by Light to Kill Paramœcia Mixed with a Watery Extract of Irradiated* 3 : 4-*Benzpyrene in Benzene. The effect of dilution and overnight contact in the dark is charted.*

Dilution of extract in mixture.	Minutes to kill under lamp.	
	Immediate.	Overnight.
1 in 2	9	6
,, 4	9	8
,, 6	9	9
,, 8	10	13
,, 10	10	10
,, 15	14	24
,, 20	19	26
,, 25	22	35
,, 30	30	35
,, 50	48	Over 60

Experiment 5.

The photodynamic potency of acridine, acriflavine, eosin and quinine sulphate was determined. These were chosen in order to compare with 3 : 4-benzpyrene. They are classical light-sensitizers and are non-carcinogenic.

A suspension of acridine in water was made up from an acetone solution. The rest were sufficiently soluble in water to be made up directly. These substances were each tested in a series of dilutions, as in Experiments 1 and 3. The results are given in Table I. It will be seen that quinine and acriflavine exhibited some toxicity in the dark. In no case did contact in the dark render the paramœcia more sensitive to light. The results also show that 3 : 4-benzpyrene is over a thousand times more potent than acriflavine, eosin and quinine sulphate. It is one hundred times more potent than acridine. It differs completely from these substances in its effect upon paramœcia after prolonged contact in the dark. The possibility arises that quinine, eosin, etc., appeared relatively weak photodynamically because of a paucity of the appropriate sensitizing light-waves in the emission spectrum of the mercury-vapour lamp. However, the same quantitative relationship was upheld when sunlight was the source of radiation.

Experiment 6.

The photodynamic potency of 1 : 2-benzanthracene, cholanthrene and methylcholanthrene was determined as in Experiments 1 and 3. Cholanthrene and methylcholanthrene are highly potent carcinogenic hydrocarbons. The results given in Table I show that these three hydrocarbons are about as active photodynamically as 3 : 4-benzpyrene. Contact in the dark increased the light sensitivity of the paramœcia in all cases. This latter effect definitely differentiates them from the acridine, eosin group.

DISCUSSION.

The mechanism of photodynamic action has been fully reviewed by Blum (1932). It is not yet fully understood. It would appear that the fluorescent dye combines with the cell protoplasm, shifting its absorption spectrum to the red end. The light absorbed by this combination produces short-lived activated molecules, some of whose energy is released for chemical change. The resultant chemical reaction, for which oxygen is necessary, has a toxic effect upon the cell protoplasm. The formation of organic peroxides is thought to play some part in this reaction.

Absorption of the colloid by the paramœcia can be observed under the microscope. The food-vacuoles are seen to become progressively stuffed with black particles when in contact with 1 in 20,000 3 : 4-benzpyrene. These particles fluoresce a bright green in ultra-violet light. The organisms can also be seen to excrete the colloid as little black pellets. Some of it must be retained. Inside the paramœcium the hydrocarbon presumably links on to the cell protoplasm ; whether in an unaltered state one cannot yet say. That the linkage of hydrocarbon to paramœcium is firm was demonstrated in the following way : Paramœcia in contact for 18 hours with 1 in 200,000 3 : 4-benzpyrene

were freed from surrounding colloid by repeated centrifuging in a large bulk of water. Samples tested after 1, 7 and 10 days were still killed by light.

In Experiments 1 and 3 above, the sensitivity of the organisms in such high dilutions of colloid is probably due to their intracellular concentration of the hydrocarbon during prolonged contact with it. In Experiment 2 the progressive increase in light sensitivity might be due to progressive intracellular storage and linkage of the hydrocarbon during the time of contact. Possibly a chemical alteration of the hydrocarbon might also be taking place.

In previous experiments (Mottram and Doniach, 1938) we found that from 3 : 4-benzpyrene in benzene irradiated in the presence of oxygen there can be extracted water-soluble products which are highly photodynamic. Experiment 4 confirms this finding. These photo-oxidation products can also be extracted from watery suspensions of 3 : 4-benzpyrene which have had prolonged irradiation by sunlight in the presence of oxygen.

In Experiments 1, 2 and 3 above, the photodynamic action of the colloid might have been due to the production of water-soluble photo-oxidation derivatives by the lamp during the exposure. Indeed, in our previous paper we assumed this to take place. This was shown not to be the case by the following experiment: Paramœcia were added to colloid previously exposed to the light for times varying from 8 minutes to 2 hours. They were then tested under the lamp. In no case was there found any reduction in the lethal time compared with that of non-irradiated colloid of an equivalent dilution. Table III shows the detailed results. It is nevertheless possible that intracellular oxidation of the 3 : 4-benzpyrene takes place inside the paramœcia, the resultant products being responsible for the photodynamic action.

TABLE III.—*The Effect upon the Light-sensitivity of Paramœcia of Adding to them an Equal Volume of a* 1 *in* 1 *million Suspension of* 3 : 4-*Benzpyrene which has been Previously Exposed to the Lamp for Various Time-periods.*

Minutes' irradiation of colloid.	Minutes to kill under the lamp.
0	8
8	8
15	7½
30	8
45	7½
60	9
120	7

Before reviewing the above data it would be well to put forward an important problem which these experiments have aroused, but not solved. Is the photodynamic action of 3 : 4-benzpyrene due to the pure hydrocarbon or to its oxidation products ? We have shown that the photo-oxidation products of the hydrocarbon are highly photodynamic. Are similar substances produced inside the paramœcia in the dark ? If so, the contact time effect could be readily explained. On general grounds we can presume that the hydrocarbon links up with the cell protoplasm. It is difficult to conceive of this linkage

unless some alteration of the hydrocarbon molecule takes place. A possible alteration is an oxidation or hydroxylation. Mottram and Doniach (1938) obtained some correlation between carcinogenicity and photodynamic activity in a small series of polycyclic hydrocarbons examined ; 1 : 2-benzanthracene was quantitatively the chief exception. This correlation might well represent ease of oxidation by the cell enzymes. That carcinogenic hydrocarbons are altered by the tissues of living animals has been demonstrated by other workers. Peacock (1936) detected a derivative of 3 : 4-benzpyrene in the bile of fowls after intravenous inoculation. Chalmers (1934), Chalmers and Peacock (1936), and Berenblum and Kendall (1936) found rapid elimination of 1 : 2 : 5 : 6-dibenzanthracene from fowls and mice inoculated in various ways. Mottram (1939) detected significant quantities of photodynamic substance in the urine of mice previously painted or inoculated with 3 : 4-benzpyrene. It is unlikely that the water-insoluble 3 : 4-benzpyrene could be rapidly eliminated from the body unless it were broken down or altered into a water-soluble derivative.

Granted the oxidation hypothesis a new problem arises. Is it the pure hydrocarbon or is it the oxidation product which gives rise to tumours ? Or is it the actual process of oxidation which arouses abnormal cellular division ? The testing of photo-oxidation products for carcinogenicity might help to throw some light on these questions.

Leaving speculation we can now review the results described. Experiments 1, 3 and 6 give a method of biological assay which detects the presence of 3 : 4-benzpyrene, 1 : 2-benzanthracene, cholanthrene and methylcholanthrene in dilutions of the order of 1 in 100 millions. Experiments 2 and 4 give a biological method of differentiating between 3 : 4-benzpyrene and its photo-oxidation products. Experiment 5 shows that the group of non-carcinogenic light-sensitizers tested was definitely less potent photodynamically than the carcinogenic agents tested in Experiments 1 and 6. They also differed qualitatively in their effect upon the light-sensitivity of paramœcia after prolonged contact in the dark.

SUMMARY.

Colloid suspensions of 3 : 4-benzpyrene in water sensitize paramœcia to light in dilutions up to 1 in 100 millions. The sensitivity of the paramœcia is markedly increased by contact with the colloid in the dark.

The photo-oxidation products of 3 : 4-benzpyrene are also photodynamic, but their action is not increased by contact in the dark.

The carcinogenic hydrocarbons 1 : 2-benzanthracene, cholanthrene and methylcholanthrene have the same action on paramœcia as 3 : 4-benzpyrene.

The non-carcinogenic light-sensitizers acridine, acriflavine, eosin and quinine sulphate are less potent photodynamically than 3 : 4-benzpyrene, and their action is not increased by contact in the dark.

The photodynamic property is a sensitive means of biological assay of the above-mentioned carcinogenic hydrocarbons.

I wish to thank Dr. J. C. Mottram, Director of Pathological Research at Mount Vernon Hospital, for his help and advice with this investigation. I

am indebted to Prof. J. W. Cook for the samples of 1 : 2-benzanthracene, cholanthrene and methylcholanthrene.

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STUDIES ON *B. PESTIS* ANTIGENS AS PROPHYLACTIC AGENTS.

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THE immunological significance of the envelope antigen of *B. pestis* has been considered by the present writer in previous papers (Schütze, 1932, 1934). It appeared that cultivation of the microbe at 37°, whether on agar on in broth, produced a vaccine richer in envelope antigen than did cultivation at 26°, and at the same time more efficient in protecting against a lethal dose of plague bacilli. The animal used in these experiments was the white rat.

More recently Sokhey has published papers (1936, 1937) in which his experimental results indicated that a broth vaccine, such as Haffkine's plague prophylactic, is superior in immunizing power when grown at 27° than when grown at 37°, and for this reason, although Sokhey made no estimation of the envelope antigen present in the two vaccines, it might be concluded that an excess of that antigen in the 37° vaccine had failed to confer an increased prophylactic potency. As the animal he employed was the white mouse, it seemed likely that this factor might explain the discrepancy in the two findings. There is indeed a notable difference in the reaction of these two animals to subcutaneous infection with virulent plague. While the mouse succumbs on an average in 8 to 9 days after a small invasive dose of one or two hundred bacilli, it takes a dose of at least one or two millions to produce a similar, approximately 100 per cent. mortality in the white rat used at the Lister Institute, death then taking place more rapidly in 3 to 5 days.

In all the writer's earlier experiments mentioned above, a virulent strain of *B. pestis* was used in the preparation of the vaccines. Otten's work on living, avirulent plague vaccines (1936, 1938) has demonstrated the existence of two types of avirulent plague cultures, which he calls the rough and the

smooth, distinguishing them both by their colonial appearances and their protective power in animals. The type which is the more efficient prophylactically he labels "smooth", although its colony possesses characters more suggestive of what is usually in other bacterial species regarded as a rough colony.

Through the kindness of Dr. Otten cultures of his strains were obtained, being sent from Java in cold storage, and so maintained without subculturing in order that no change in their characters might ensue. The cultural distinctions as described by Otten have been observed. Smooth cultures grown at 37° for three days present colonies less shiny of surface and more opaque than do rough cultures; the margin of a smooth colony is crenellated; the flat and spreading fringe is in marked contrast to the heaped interior; the four smooth strains Tjiwidej S, Java S, Soemedang and Bombay Rat agreed in these respects with all virulent strains examined; on the other hand, the two rough strains Tjiwidej R and Java R have colonies with margins that may be irregular in outline but are normal confines to a translucent colony, and not fringes strongly differentiated from an opaque interior. In broth at 37° a distinction between rough and smooth is less easily demonstrated though, examined under a lens, the smooth avirulent, like the virulent cultures, exhibit a turbidity slightly more homogeneous than that of the rough cultures. Tested for sensitivity to the oxidation-reduction potential of media by seeding thinly on to agar with and without the addition of Fildes's peptonized blood, all plague cultures, whether virulent or avirulent, rough or smooth, with one exception exhibit an oxygen sensitiveness such as has already been described (Schütze and Hassanein, 1929). Such oxygen sensitivity on the part of rough cultures is not in accordance with the findings of Webster (1925) and Webster and Baudisch (1925) working with the pasteurellas. Rough variants in this species grew equally well whether the oxygen-reduction potential of the medium was adjusted by the addition of a reducing substance or not. In this respect, therefore, Otten's rough plague strains do not conform to rough variants among the pasteurellas. His avirulent strains behave rather as if all except one were equally smooth. This one exception, which was insensitive to oxygen, was a smooth strain—Java S. Thinly seeded over agar plates it grew equally well whether peptonized blood was present or not. A lack of sensitivity on the part of smooth pasteurella cultures has already been noted by Schütze and Hassanein; it was never so complete as in the case of Java S.

Apart from the colonial appearances already discussed and the question of virulence, the rough and smooth avirulent cultures could not be distinguished biologically or serologically from one another or from the virulent forms. All three develop envelopes at 37° and react similarly with envelope antibody. No antigens responsible for smoothness or virulence could be detected serologically, by agglutination, precipitin or absorption tests.

A variant, however, occurred spontaneously in one of the rough avirulent cultures, Tjiwidej R. Colonies of a larger, grosser form were noted, and on isolation proved to be devoid of envelope antigen. The strain neither reacted with envelope antibody, nor was capable of inducing it after inoculation into the rabbit. In every other respect this unenveloped variant, hereafter called TRU, resembled its parent form. At a subsequent period the remaining

rough avirulent culture, Java R, was seen to have produced an unenveloped variant, but so far none of the smooth forms has been found to throw variants lacking the characteristic envelope antigen.

In the absence of envelope and consequently of one of the chief characters distinguishing *B. pestis* from *B. pseudotuberculosis rodentium*, and to exclude the possibility of this unenveloped strain being a contaminating organism of that species, special attention was paid to other differentiating tests. The variant TRU was non-motile at all temperatures, failed to ferment rhamnose, failed to produce alkalinity in litmus milk, and was sensitive to the oxygen-reduction potential of the medium. It could, therefore, be regarded as a genuine plague strain, lacking, however, the power of forming envelope substance. This strain was included in later immunity experiments; in the earlier ones its presence in the parent culture was unsuspected, but, doubtless, served to explain otherwise unaccountably low envelope titres in the Haffkine vaccines prepared with that culture.

The following experiments were planned to demonstrate what correlation there might be between the envelope content of a vaccine and its prophylactic efficiency in both rat and mouse. The avirulent rough and smooth strains were included for the purpose of investigating what benefit smoothness or virulence might confer on a Haffkine vaccine.

DETAILS OF EXPERIMENTS.

The Haffkine vaccines were grown in broth, pH 7·6, in flasks filled to a depth of about 2 cm. and incubated for 4 weeks at a temperature of either 26° or 37°; after being subcultured to establish purity, the flasks were heated to 55° C. for 15 minutes, and on cooling phenolized to 0·5 per cent. The method of preparation thus conformed to that employed by Sokhey, and in later experiments the culture medium was an acid digest broth as used at Parel. Included in the experiments were Haffkine vaccines very kindly prepared and sent by Lt.-Col. Sokhey, along with virulent strains for test dose purposes. By titrating the supernatant of centrifuged samples of these vaccines against a constant envelope antibody solution, it was possible by means of the precipitin test to assess their envelope (E) content.

The vaccine was administered in two subcutaneous doses at an interval of 7 days, the first being half the size of the second.

After a further interval of 14 days the test dose was given subcutaneously. In rats it consisted of ½ c.c. of undiluted 2-day 37° broth culture of a virulent plague strain, and contained about 10 million viable bacteria as established by plate counts.

In mice the test dose was ½ c.c. of a 2-day 26° broth culture diluted 1/200,000 or 1/500,000, and containing roughly 200 or 100 living organisms. The experiment was allowed to run in the case of rats for 14 days and of mice for 21 days.

In each group of control rats or mice one or two animals usually survive. As figures taken from virulence tests indicate, these survivals are dependent upon an exceptional natural immunity in those individuals. For example, given a dose of 3 million, 1 out of 18 rats survived, while given ten times that amount still, 1 survived out of 23 inoculated; in mice 40 organisms killed

21 out of 23 animals, while more than six times that amount still killed only 21 out of 24.

Rat and Mouse Experiment I.

Vaccine prepared with a virulent strain and grown at 37° was compared with a similar one grown at 26° and one prepared with the avirulent smooth strain Tjiwidej S grown at 37°. The envelope titre for both 37° vaccines was 1/16, while that for the 26° vaccine was only 1/4, that is to say, the supernatants of the 37° vaccines could be diluted 16 times and still react in the precipitin test with envelope antibody, while the 26° vaccine did not do so when diluted more than 4 times.

TABLE I.—*Haffkine Vaccines.*

Animals.	Strain used.	Grown at—	E Titre.	Total dose (c.c.).	Test dose.	Number of animals.	Percentage survivors.
Rats	Virulent	37°	1/16	3/8	10×10^6 organisms	21	95
	Avirulent Smooth	37°	1/16	3/8	Ditto	21	86
	Virulent	26°	1/4	3/8	,,	21	62
	Controls	..	..	..	,,	21	10
Mice	Virulent	37°	1/16	1/60	258 organisms	24	54
	Avirulent Smooth	37°	1/16	1/60	Ditto	23	65
	Virulent	26°	1/4	1/60	,,	23	52
	Controls	..	..	..	,,	24	12

Table I indicates that *in the rat* a relationship between envelope (E) titre and prophylactic potency exists; an avirulent smooth culture grown at 37° gives as good an E titre as a virulent culture at that temperature and protects as well, while a virulent culture grown at 26° has a lower E titre and protects less well; *in the mouse*, on the other hand, E titre does not appear to influence prophylaxis any more than does virulence. A diminution in the envelope content to a quarter does not lower the protective value of the vaccine. An avirulent culture protects as well as a virulent one.

Rat Experiments.

Rat Experiment II.

In this experiment two vaccines prepared from the smooth avirulent strains Soemedang and Tjiwidej S were compared with one made from the rought avirulent strain Tjiwidej R; all were grown at 37°, but the rough strain yielded a very poor E titre which was doubtless due to the presence in the culture of the unenveloped variant TRU, already mentioned. Subsequent vaccines prepared from the culture after elimination of the variant gave normally high E titres, while one made with TRU contained no envelope substance at all.

TABLE II.—*Haffkine Vaccines in Rats.*

Strain used.	Grown at—	E titre.	Total dose (c.c.).	Test dose.	Number of animals.	Percentage survivors.
Avirulent Smooth (Soemedang).	37°	1/24	3/16	30×10^6 organisms	20	85
Avirulent Smooth (Tjiwidej S)	37°	1/16	3/16	Ditto	21	86
Avirulent Rough (Tjiwidej R)*	37°	1/3	3/8	,,	21	57
Controls	..	..	..	,,	21	5

* The culture was largely composed of the envelopeless variant, TRU.

Table II demonstrates that a fall in the E titre from 1/24 or 1/16 to 1/3 occasions a marked diminution in protective efficiency of a vaccine. Even though the immunizing dose of Tjiwidej R is double that of the two other vaccines, the percentage of survivors is notably smaller. That this is not due to lack of smoothness in the culture is demonstrated by the next experiment.

Rat Experiment III.

Vaccines prepared from the smooth and rough Tjiwidej strains, the latter now freed from the envelopeless variant with which it was contaminated, were compared. Half of the animals were subjected to a smaller test dose than that usually employed.

TABLE III.—*Haffkine Vaccines in Rats.*

Strain used.	Grown at—	E titre.	Total dose (c.c.).	Test dose.	Number of animals.	Percentage survivors.
Avirulent Smooth	26°	1/6	3/16	13×10^6 organisms	15	47
Avirulent Rough	26°	1/12	3/16	Ditto	15	47
Controls	..	..	..	,,	15	0
Avirulent Smooth	26°	1/6	3/16	$0{\cdot}4 \times 10^6$ organisms	15	72
Avirulent Rough	26°	1/12	3/16	Ditto	14	64
Controls	..	..	..	,,	15	27

Table III shows that a normal envelope-developing rough culture yields a vaccine with normal E content and as protective as one made from a smooth culture.

Rat Experiment IV.

To demonstrate that the envelopeless variant TRU is deficient in prophylactic power, a vaccine prepared therefrom was contrasted with one made with a normal envelope-producing strain.

TABLE IV.—*Haffkine Vaccines in Rats.*

Strain used.	Grown at—	E titre.	Total dose (c.c.).	Test dose.	Number of animals.	Percentage survivors.
Envelope producing	37°	1/32	3/16	20×10^6 organisms	20	70
Envelopeless	37°	0	3/16	Ditto	21	4
Controls	..	..	..	,,	21	12

In Table IV is seen that the envelopeless variant TRU grown at 37° like the envelope-producing strain furnishes a vaccine devoid of envelope substance, and devoid, too, of any protective power.

E Titre and Plague Prophylaxis in the Rat.

In all four experiments a positive correlation between the E titre of a Haffkine vaccine and its prophylactic efficiency in the rat has been established. This result confirms those arrived at by the writer in work previously published (Schütze, 1932). At the same time no difference in prophylactic efficiency was noticeable whether the strain used in its manufacture was virulent or avirulent, rough or smooth. In all four types envelopes are equally well developed, and Haffkine vaccines made therefrom have similar E titres and protect equally well.

Mouse Experiments.

In Experiment I it was seen that with the mouse as the test animal, no difference in prophylactic value could be detected between vaccines made from a virulent or an avirulent smooth culture, nor did a fall in the E titre to 1/4, occasioned by growth at 26°, result in a less efficient vaccine.

Mouse Experiment V.

In the ensuing Experiment V, vaccines prepared from two avirulent smooth strains, Tjiwidej S and Soemedang, were compared with one made from an avirulent rough strain, Tjiwidej R. All were grown at 37°, but Tjiwidej R vaccine had a particularly low E titre because of the preponderance of the unenveloped variant, TRU, as already stated.

TABLE V.—*Haffkine Vaccines in Mice.*

Strain used.	Grown at—	E titre.	Total dose. (c.c.).	Test dose.	Number of animals.	Percentage survivors.
Avirulent Smooth (Soemedang).	37°	1/24	1/60	260 organisms	24	54
Avirulent Smooth (Tjiwidej S) .	37°	1/16	1/60	Ditto	24	42
Avirulent Rough (Tjiwidej R)*.	37°	1/3	1/60	,,	23	30
Controls	..	..	..	,,	24	5

* The culture was largely composed of the envelopes variant, TRU.

In this experiment an influence of E titre on prophylaxis in mice is indicated. Soemedang vaccine possesses an E titre 8 times that of Tjiwidej R, and at the same time seems significantly more effective as immunizing agent.

Mouse Experiment VI.

A further test of E titre influence was made by comparing vaccines grown variously at 37° or 26°, the culture being the same virulent strain throughout.

TABLE VI.—*Haffkine Vaccines in Mice.*

Strain used.	Grown at—	E titre.	Total dose (c.c.).	Test dose.	Number of animals.	Percentage survivors.
Virulent	37°	1/24	1/60	66 organisms	24	54
Virulent	26°	1/8	1/60	Ditto	23	39
Virulent	26°	1/4	1/60	,,	23	35
Controls	..	..	..	,,	23	4

From Table VI one sees that growth at 26°, resulting in a lowered E titre, also produces a vaccine which is less protective for mice.

Mouse Experiment VII.

As both the last mouse experiments indicated that E titre influences plague prophylaxis in mice, while Experiment I did not, a further experiment was devised in which a larger number of vaccines with varying E titres, and prepared from a variety of strains, were compared. Besides vaccines prepared at the Lister Institute from a virulent strain grown at 37° and 26°, through the kindness of Lt.-Col. Sokhey, similar Haffkine vaccines made at Parel were included. The avirulent strains Tjiwidej S and Tjiwidej R were grown at 37°, as was the unenveloped variant TRU. All vaccines grown at 37° gave E titres of 1/32 or 1/16 except TRU, which possessed no envelope antigen ; vaccines grown at 26° or 27° gave E titres of 1/8. Except in the case of TRU the difference in E titre is, therefore, not pronounced. On this occasion the broth taken for the Lister Institute Haffkine was an acid digest broth, brought from Parel by Dr. P. B. P. Naidu some years previously. Immunization was carried out in one series with the usual total dose of 1/60 c.c. per mouse, and in a second series of animals with one quarter that amount. The results are given in Table VII.

TABLE VII.—*Haffkine Vaccines in Mice.*

Strain used.	Grown at—	E titre.	Total dose. (c.c.).	Test dose.	Number of animals.	Percentage survivors.
Virulent	37°	1/32	1/60	91 organisms	24	33
Parel	37°	1/16	,,	Ditto	22	27
Avirulent smooth	37°	1/16	,,	,,	23	43
Avirulent rough	37°	1/16	,,	,,	24	54
Virulent	26°	1/8	,,	,,	23	27
Parel	27°	1/8	,,	,,	24	38
TRU	37°	0	,,	,,	24	29
Virulent	37°	1/32	1/240	,,	23	22
Parel	37°	1/16	,,	,,	23	13
Avirulent smooth	37°	1/16	,,	,,	23	32
Avirulent rough	37°	1/16	,,	,,	24	50
Virulent	26°	1/8	,,	,,	23	26
Parel	27°	1/8	,,	,,	24	33
TRU	37°	0	,,	,,	24	0
Controls	..	..	..	,,	24	4

The results obtained in this experiment and recorded in Table VII indicate

that envelope antigen by no means plays the important part in immunizing mice that it does in rats. There is no consistent association between height of envelope titre and degree of protection. Vaccine TRU, when administered in the usual dose of 1/60 c.c., though devoid of envelope antigen, is nevertheless capable of protecting as well as the Parel Haffkine also grown at 37°, which has an E titre of 1/16. One is forced to conclude that some further antigen must be concerned in establishing immunity in the mouse. This antigen might be irregularly present in the various strains or suffer damage irregularly in the course of vaccine preparation, which would account for the dissimilarity in prophylactic potency of the various batches and explain, for instance, the unexpected superiority of the vaccine prepared from the rough avirulent strain.

Such variation in the efficacy of this antigen would tend to obscure whatever influence on immunity envelope antigen might possess in the mouse. For this reason, it should be more useful to compare the average protection afforded by a number of vaccines of higher E titre with that achieved by a similar number with lower E titres. In all the experiments detailed above, some 23 vaccines have been tested on 483 mice ; 273 of these animals were inoculated with 13 vaccines whose E titres were 1/16 or more, while 210 animals were inoculated with 10 vaccines whose E titres were 1/8 or less.

The group of vaccines containing the larger amount of envelope antigen protected 42 per cent. of the mice, while the group containing the smaller amount protected only 31 per cent. In view of the number of vaccines tested and of mice employed these figures would seem to be significant, and to indicate that in the mouse, too, envelope antigen is of immunological importance, though not so outstandingly as it is in the case of the rat.

On the other hand, it has not been possible to demonstrate immunological superiority in a virulent strain over an avirulent one or in a smooth strain over a rough one. This would appear to contradict work done by Otten (1936) who has plainly shown how important for protective vaccination the antigenic condition of a culture is, but one must remember that he was using living vaccines. With these living avirulent cultures he found that survival rates could be increased as much as three or four times by substituting a smooth for a rough culture.

An outstanding difference in the two types of innoculation is the fact that in using a killed vaccine one gives a definite amount of preformed antigen, while with a living vaccine one relies upon its manufacture in the animal's own body, and the extent to which this manufacture is possible may well depend on the length of time the strain is able to persist and grow in the animal.

Comparison of Rough and Smooth Living Vaccines.

To test this point, guinea-pigs were inoculated intraperitoneally with $\frac{1}{2}$ c.c. of living suspensions of the avirulent cultures Tjiwidej Smooth and Tjiwidej Rough containing 1000×10^6 organisms. After periods varying from 3 to 11 days the animals were killed, and the omentum and spleen examined for viable plague bacteria by incubating those organs in broth at 37°. It was then seen that 75 of such examinations carried out on guinea-pigs inoculated with

the smooth variant yielded 48 cultures of plague, whereas 72 comparable examinations on guinea-pigs inoculated with the rough variant gave 18 cultures, that is to say, the smooth organism was found in 64 per cent., the rough only in 25 per cent. of the examinations. This demonstration of the living smooth variant's greater survival power was followed by a series of experiments devised to test its efficacy as an envelope antibody-producing inoculum.

Rabbits were inoculated intravenously with living cultures of Tjiwidej, 20 with the smooth variant, 20 with the rough. The doses, given in three portions at 6 days' interval, varied in different experiments from 5×10^6 to 40×10^6 organisms; a week after the last inoculation the animals were bled and the envelope titres of the sera established by the precipitin test. Two similar groups of rabbits were given killed cultures of the two variants, 750×10^6, 1500×10^6 and 3000×10^6 organisms at 6 days' interval and titrated for envelope antibody 7 days after the last inoculation. The results obtained are given in Table VIII.

TABLE VIII.—*Envelope Antibody Production after Vaccination with Smooth and Rough Avirulent plague.*

Vaccine.	Number of rabbits giving precipitin titres of								Geometric mean of titres.
	<1/2.	1/2.	1/4.	1/8.	1/16.	1/32.	1/64.	1/128.	
Living Tjiwidej Smooth	0	0	1	4	6	6	3	0	1/21
,, ,, Rough	2	2	3	4	5	4	0	0	1/8
Killed Tjiwidej Smooth	2	2	3	3	3	6	2	0	1/12
,, ,, Rough	3	0	1	6	3	5	2	1	1/13

It is clear from these results that given in the form of a living inoculum the smooth variant produces on the average a higher E titre than does the rough variant. This superiority is doubtless dependent on its capacity for more prolonged growth in the animal body, with consequent greater opportunity for elaboration of envelope antigen. When the inoculum is administered in killed form, the amount of envelope antigen is similar in both vaccines and results in the appearance of similar E titres in each group.

DISCUSSION.

It has always been assumed that in the preparation of a Haffkine plague vaccine, a highly virulent strain of *B. pestis* is essential. In the experiments recorded in this paper, vaccines made from virulent, smooth avirulent and rough avirulent strains have been compared. All were prepared in similar fashion. Growth took place in broth for 4 weeks, and sterilization was effected by heating to 55° C. for 15 minutes, phenol to 0·5 per cent. being subsequently added. Two series of experiments were carried out, one in which the test animal was the rat and the test dose a large toxic one of ten or more million bacteria, the other in which mice were employed and given a small invasive test dose of one or two hundred bacteria. No superiority of virulent strains over avirulent or smooth over rough could be demonstrated. All were capable of producing vaccines of equal merit. For maximal protection of the rat it is essential that the vaccine be grown at 37°. This confirms previous work, and is explained by the fact that at higher temperatures more envelope antigen develops in a plague culture, and that this antigen is of considerable prophylactic

importance in the rat. In the protection of the mouse, envelope antigen appears to play a smaller part. There is not the same correlation between envelope antigen content of a vaccine and its protective power. It must be some other antigen which principally influences prophylaxis in the mouse, and it must, like envelope antigen, develop equally well in avirulent as in virulent, in rough as in smooth, cultures.

This observation appears to contradict the results obtained with living avirulent vaccines in plague prophylaxis, where it is essential that the strain employed should be a smooth one. It has, however, been shown that at least so far as the envelope antigen is concerned, and possibly for other antigens as well, the superior prophylactic power of a smooth avirulent strain given in the form of a living vaccine depends on its capacity for prolonged survival in the inoculated animal, with consequent increased production and liberation of antigen. A rough strain is more quickly suppressed by the defensive mechanisms of the animal and has, therefore, less time in which to elaborate antigen in the body of that animal.

SUMMARY.

1. Plague prophylaxis by means of heat-killed broth vaccines depends, in the rat, where the test dose is a large and toxic one, markedly on the presence in the vaccine of envelope antigen.

2. As this antigen is more abundantly produced at higher than at lower temperatures, vaccines grown at 37° C. are more effective than those grown at 26° C.

3. In the mouse, where the test dose is a small and invasive one, the effect of envelope antigen is less pronounced.

4. Virulent cultures do not result in more potent vaccines for either rats or mice than do avirulent ones, nor are smooth cultures more effective than rough ones, when these vaccines are killed.

5. It has been shown that a smooth avirulent culture used in the form of a living vaccine survives longer in the guinea-pig and produces a higher envelope antibody titre in the rabbit than does a rough avirulent culture. This would explain the apparent discrepancy between the statement in paragraph 4, and the well-known fact that the smooth variant is superior to the rough in the making of living avirulent vaccine.

6. No serological distinction was established between virulent and avirulent, smooth and rough, forms.

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THE ANTIGENIC PROPERTIES OF STREPTOLYSIN.

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A SERIES of investigations was started some years ago with a view to throwing some light on the curious paradox exposed by Todd's work (1932). Todd has shown that the hæmolysin produced by streptococci in serum-free media is antigenic, whereas that produced in media containing a considerable percentage of serum is not. In respect of the second finding Todd's results are entirely in line with much previous work.

It had been found that with the strains at our disposal, and using a medium which only diverged from that of Todd and Hewitt (1932) in the respect that the peptone was incorporated with the meat at the time of extraction, as recommended by Hedley Wright (1933), we were able to get hæmolysins more powerful than those described by Todd, without requiring to resort to reduction of the filtrates. Our first investigation was devised to find out whether such non-reduced serum-free hæmolytic filtrates were, like his, antigenic. The first investigations made did definitely suggest the production of antibody, and further there appeared to be some connection between the antihæmolytic titre of a rabbit's serum and the animal's ability to resist the lethal effects of large doses of such toxins. This conclusion appeared to be one of some possible importance, and an extended investigation was planned to test it. In the course of trying out a series of modifications of medium it was found that one prepared with meat extracted at a lower temperature than the boiling-point, yielded with some strains at any rate, and with one in particular, cultures which could be filtered without serious loss of hæmolytic activity.

It was noted also that these filtrates exhibited a quite unusual toxicity to rabbits, the best attained killing in a dose of 1 c.c. or less, even when administered intramuscularly. This, so far as we know, is a more powerful toxin than any unconcentrated streptococcal toxin which has so far been described, with the exception of some obtained long ago by Marmorek (1902) from strains subjected to long series of passages.

EXPERIMENTAL.

Medium Employed.

To 1 lb. of mince add 1 litre of tap-water; leave overnight in the cold room, temperature $-2°$ to $2°$ C. Next morning add 20 g. Difco proteose peptone and mix well. Extract in a cold water bath by raising the temperature gradually until it rises to $75°$ C at the end of four hours.

Filter through lint, and add sufficient $N/1$ NaOH to bring the reaction to pH 7·4. Allow to stand for a few minutes. Filter through Chardin filter-paper while still warm, and add the following ingredients to the filtrate :

0·20 per cent. glucose.
0·20 ,, sodium bicarbonate.
0·10 ,, di-sodium phosphate.
0·20 ,, sodium chloride.

Adjust the reaction to pH 8·0 and filter through Chamberland candles. Deliver aseptically into (7″×1″) tubes, about 40 c.c. each tube.

Reaction.—Reactions are determined with small samples of the medium which have been boiled to coagulate the proteins and then filtered. The original medium is too highly coloured to lend itself to accurate colorimetric determination of pH.

Strains of Streptococcus used.

A dozen different strains were tried out in all. These included a few stock strains which had been in subculture for some years, but the majority were strains recently isolated from various types of infection, amongst which streptococcal sore throats predominated. A number of these were approximately equal in the hæmolytic activity of their cultures after 12 to 14, hours' incubation, which was usually the period of greatest activity. One, however, a strain isolated from the throat in a case of scarlatina, was superior to the others in the respect that its cultures could usually be filtered (Seitz filtering disc or Maassen candle) with very little loss of hæmolytic activity.

This strain fell into Lancefield's group A.

Hæmolytic Titres of Cultures and Filtrates.

The best titres ever obtained were such that the minimum hæmolytic dose, as tested against 1 c.c. of 2 per cent. washed ox blood, and on reading after 2 hours' incubation followed by 2 hours at room temperature, was 0·005 c.c. or slightly below it.

The titres of the filtrates varied from 0·005 c.c. to 0·5 c.c. The variation seemed to be determined (*a*) by differences in the strains, and (*b*) by differences in the batches of medium. Even the strain used throughout these investigations became less constant in yielding powerfully hæmolytic filtrates towards the end of the investigation when it had been three years in subculture.

Effects of Reduction of Filtrates.

Reduction was carried out according to the technique of Todd (1932) by adding 1 per cent. of $N/1$ NaOH and 0·1 per cent. of $Na_2S_2O_4$ and leaving the filtrate standing in an evacuated vessel. The results varied a good deal, but can best be summed up by saying that filtrates much inferior in hæmolytic activity to the cultures from which they had been obtained usually had their hæmolytic activity increased, and in a few cases it was restored to that of the unfiltered culture. In no case, however, was a hæmolytic titre obtained by means of reduction superior to that of the best filtrate obtained without reduction.

Results of Concentration of Filtrates.

A modification of Craigie's (1931) method of drying *in vacuo* from the frozen state was employed. Volumes of 50–80 c.c. were dried in this way, and then taken up in a volume of distilled water equal to one-sixth to one-tenth of the original bulk.

It was found that marked increases of hæmolytic activity were obtained in this way which were approximately equivalent to the concentrations in bulk, and in this respects these results corresponded to those obtained by Channon and McLeod (1929) with serum hæmolysin.

Natural Antihæmolysin to Serum Hæmolysin and Serum-free Hæmolysin Compared.

Before proceeding to consider the effect of immunization of animals with hæmolytic toxin it is desirable to know the range of normal variation in antibody content of animal sera. With serum hæmolysin this had been done previously for rabbit sera by McLeod and McNee (1913) and Channon and McLeod (1929), but a comparison between the neutralizing power of normal sera for serum hæmolysin and serum-free hæmolysin respectively had not been made. The sera of 50 rabbits were compared, their antihæmolytic titres being determined for single doses of hæmolysin. A second series of 54 rabbits was examined, their antihæmolytic titres being determined for 1 and 2 M.H.D. of serum-free hæmolysin and of serum hæmolysin.

TABLE I.

Rabbit No.	Serum-free filtrate.			Serum filtrate.	
	1 M.H.D. (c.c.).	2 M.H.D. (c.c.).	3 M.H.D. (c.c.).	1 M.H.D. (c.c.).	2 M.H.D. (c.c.).
52	0·15	0·20	0·30	0·08	0·15
53	0·02	0·05	T.H. 0·10	0·08	0·15
54	0·05	0·10	T.H. 0·20	0·10	0·20
60	0·02	0·05	0·10	0·15	0·30
63	0·10	>0·20	>0·30	0·10	0·20
83	0·15	>0·20	>0·30	0·05	0·10
87	0·12	T.H. 0·20	>0·30	0·08	0·15
90	>0·15	>0·20	>0·30	0·10	0·20
104	0·02	0·05	0·10	0·10	0·20
105	0·10	T.H. 0·20	T.H. 0·30	0·20	0·30

T.H. = Trace hæmolysis.

Note.—Figures in columns indicate amounts of serum required to neutralize single or multiple doses of serum streptolysin and glucose-broth streptolysin respectively.

A selection from these results is given in Table I. It shows that the antihæmolytic activity is usually greater for serum-free hæmolysin than for serum hæmolysin; also that there is a greater individual variation in the activity of the sera in respect of their power of neutralizing serum-free hæmolysin than is observed with serum hæmolysin.

In so far as they go, the results would favour the hypothesis that serum-free hæmolysin and serum hæmolysin are distinct substances, since one serum may be found to be active in neutralizing serum-free hæmolysin and weak against serum hæmolysin, while another gives the opposite result—for example the sera of rabbits 83 and 60 in Table I.

Experiments on the Immunization of Rabbits.

The earlier and more limited series of experiments had indicated that (*a*) rabbits could produce antihæmolysin when injected with a hæmolysin produced in the special medium described, and differing from Todd's hæmolysin in the respect that it usually retained its activity for many hours after filtration, without having recourse to reduction of the filtrate, and (*b*) that animals so immunized were more resistant than normal animals to the lethal effects of the hæmolytic toxin, a definite relationship existing between the antilysin content of the animals' serum and the resistance of the animal to lethal toxin.

In order to test these tentative findings more carefully, 60 rabbits were chosen and split into 5 groups :

A consisted of 12 fresh animals which were injected intramuscularly with filtrate of serum-free hæmolysin obtained from 14-hour cultures of the strain of streptococcus selected. The scheme of inoculations, quantities and intervals of time is given in Table II. The filtrates were not reduced.

TABLE II.—*Scheme of Immunization—Control Series D.*

Rabbit No.	Date.	Dose.
82	2.xi.36	3 c.c. I.M. of heated serum-free filtrate of S6.
	16.xi.36	1·5 ,, ,, ,, ,, ,,
	23.xi.36	2 ,, ,, ,, ,, ,,
	28.xi.36	3 ,, ,, ,, ,, ,,
	2.xii.36	4 ,, ,, ,, ,, ,,
	7.xii.36	5 ,, ,, ,, ,, ,,
	11.xii.36	5 ,, ,, ,, ,, ,,
	16.xii.36	5 ,, ,, ,, ,, ,,
	4.i.37	8 ,, ,, ,, ,, ,,
	12.i.37	10 ,, ,, ,, ,, ,,
	19.i.37	8 ,, ,, ,, ,, ,,
	26.i.37	10 ,, ,, ,, ,, ,,
	30.i.37	10 ,, ,, ,, ,, ,,

I.M. = Intramuscular.

B consisted of 12 rabbits which had survived from the earlier experiments, and which might be considered to be already partially immunized. These were treated in the same way as Group A, except that in the case of 5 of them the last three or four injections were made with reduced filtrates.

C consisted of 12 fresh rabbits which were treated with serum hæmolysin, the dosage, etc., being similar to that given to Group A. The medium used was meat extract broth containing 20 per cent. of inactivated horse-serum.

The object of including this third group was to test Todd's statement that serum hæmolysin, although unneutralized by the sera of animals prepared with either serum or serum-free hæmolysin, was nevertheless capable of stimulating the production of antibody to serum-free hæmolysin, *i. e.* it acted as antigen calling forth an antibody to the action of which it was insensitive.

D. This was the first control series, and consisted of 12 rabbits injected with medium only, the dosage, etc., being as in the other series.

E. The second control series consisted of 12 rabbits which received injections of the same filtrates as were used in Series A, but which had been heated 30 minutes at 60° to 65° C. to destroy the hæmolysin.

The same strain of streptococcus was used to inoculate all cultures.

Antihæmolytic Activities of Immunized and Control Rabbits' Sera.

Five weeks after the last injection of the series blood was drawn from two or three rabbits in each group, and the antihæmolytic titres of the sera were determined for serum-free hæmolysin and serum hæmolysin respectively.

The method of carrying out these tests and the full results with one serum in each category is given in Table III for serum-free hæmolysin. When the number of M.H.D. neutralized by 1 c.c. of serum is calculated on the basis of the observations recorded in Table III, the comparison of the different sera tested is rendered easier, and the results so obtained are set out in Table IV.

TABLE III.

Number of M.H.Ds. of toxin.	Number of antiserum and its series.	Amounts of antitoxins and residual hæmolytic activity after incubation of toxin and antitoxin for 30 min.			
		0·1 c.c.	0·05 c.c.	0·01. c.c	0·005 c.c.
2½	75A	—	—	—	—
5	,,	—	—	—	C.*
7½	,,	—	—	C.	C.
2½	648B	—	—	—	—
5	,,	—	—	—	—
7½	,,	—	—	—	C.
2½	88C	C.	C.	C.	C.
5	,,	C.	C.	C.	C.
7½	,,	C.	C.	C.	C.
2½	97D	C.	C.	C.	C.
5	,,	C.	C.	C.	C.
7½	,,	C.	C.	C.	C.
2½	70E	C.	C.	C.	C.
5	,,	C.	C.	C.	C.
7½	,,	C.	C.	C.	C.

* = Complete hæmolysis.

Results of titrations for antihæmolysin to serum-free hæmolysin only are given in this table.

TABLE IV.

Rabbit No.	Group.	Number of neutralizing doses per c.c. against serum-free hæmolysin.	Number of neutralizing doses per c.c. against serum hæmolysin.
59	I immunized with glucose broth hæmolysin	600	..
75		550	20
95		..	20
648	II re-immunized as last	>600	..
708		>1000	20
54	III immunized with serum hæmolysin	30	20
70		<25	20
97	IV control received medium only	<25	..
98		20	20
103		20	..
86	V control received, heated glucose broth hæmolysin	20	20
88		<25	..

The number of rabbits investigated was small; the results, however, fall into line with those obtained earlier. The inhibition of hæmolytic activity in the sera of the immunized rabbits exceeded that observed even in the most active of any of a very large series of normal rabbits (more than 100) investigated previously. In this series the number of neutralizing doses per c.c. of serum had ranged from 5 to 40 for serum-free hæmolysin and from 5 to 20 for serum hæmolysin.

It is clear, therefore, that Todd's (1932) contention that serum-free hæmolysin stimulates antibody formation and is neutralized by the antibody formed is fully confirmed by these observations, even although the method of obtaining the serum-free hæmolysin was different from his and a medium much richer in precipitable proteins was used. The observations also come into line with those so frequently repeated in the past, in which serum hæmolysin does not produce antibody by which it is neutralized.

The results, however, in Group C do not confirm Todd (1933) in his contention that serum hæmolysin acts as an antigen in stimulating production of antilysin for serum-free hæmolysin.

Development of Immunity to Lethal Toxin in Rabbits Immunized by Serum-free Hæmolysin.

At varying intervals after the tests for antihæmolytic activity had been performed, batches of these rabbits containing two or more from each group were inoculated at the same time with a dose of a culture filtrate which was thought likely to be lethal to normal rabbits. Each batch so inoculated included two or more fresh animals as additional controls.

The results obtained in these observations are recorded in Table V.

A considerable number of animals were lost in one way and another, some dying as a result of the first immunizing injections, which were too large, and one group because the dose used was too small to kill any of the controls.

TABLE V.

Controls.

Category.	Number injected.	Number which died.	Percentage dying.
Fresh animals . .	11 .	8 .	..
Animals injected with medium only . .	7 .	4 .	..
Animals injected with heated filtrate . .	8 .	5 .	..
Total controls .	26 .	17 .	65·4
Immunized.			
Receiving serum-free hæmolysin . .	22 .	3 .	13·4
Receiving serum hæmolysin . .	8 .	4 .	50·0

The final results were only obtained on 75 per cent. of the group of animals with which the experiment was launched.

It is clear, however, that a considerable degree of immunity had developed from the injection of the filtrates containing serum-free hæmolysin whether the hæmolysin had played a part or not. This immunity, however, was limited in extent, since three of the " immunized " animals died.

The fact that 30 per cent. of the controls survived is probably to be ascribed to a very varied susceptibility of the animals. This has repeatedly shown itself, since the death of all the controls (fresh animals) in any experiment has been a rare phenomenon when a considerable number of control animals has been included, and in several experiments it had been observed that amongst several controls the animal or animals receiving the larger doses have survived.

These results are consistent with those obtained previously in a smaller series, and indicate at least a strong probability that the lethal toxin identical with or associated with the hæmolysin in these filtrates does evoke an active immunity in the animals to which it is injected. It is possible that the evidence of immunity resulting from toxin injection would have been more convincing if irregularities due to varying susceptibility of the rabbits had been excluded.

No attempt was made in the experiment recorded above to work entirely with one breed of rabbit. Pulvertaft (1929), however, has drawn attention to the special susceptibility of chinchilla rabbits to the concentrated streptococcal toxins which he used. In view of this a second and similar series of experiments was launched in which the rabbits used were mainly chinchillas. This series proved ill-starred in every respect. The chinchillas were difficult to immunize, many dying in the course of the preparatory injections, some apparently directly from the effect of the toxin, and others owing to intercurrent bacterial disease, notably pneumococcal infection. And finally, when the surviving and immunized animals were tested for resistance to lethal toxin with controls, the results obtained amongst the controls were so irregular that

little reliance could be placed on the evidence of immunization in the experimental animals. One point, however, did emerge from this series of investigations, which was that the antihæmolytic titres of the sera of the control animals showed a surprisingly wide variation, and this may well have accounted for the irregularity of the results when they were used as controls in determining the lethal effect of the toxin used on the immunized animals. These irregularities are recorded in Table VI, and the lethal effects of injections of toxin are also indicated in those instances in which such injections were carried out in comparable conditions. It is obvious that there has been a considerable although incomplete correlation between antihæmolytic titre as determined beforehand, and resistance to the lethal effect of the filtrate of streptococcal culture.

TABLE VI.—*Variation in Antistreptolysin Content of "Normal" Rabbits and Resistance to Lethal Toxin.*

Rabbit No.	Antistreptococcal titre.	Result of injection of lethal toxin.	Rabbit No.	Antistreptococcal titre.	Result of injection of lethal toxin.
425	6000	S	422	75	S
424	4850	S	429	75	D—2 days
496	1100	S	420	70	S
499	500	D—3 weeks	503	30	D—2 days.
501	400	D—24 hours	430	25	S
433	375	S	436	25	D—10 days.
435	250	D—3 days	419	<16	S
434	160	S	439	<16	D—24 hours.
418	120	S	440	<16	D—2 days.
500	120	D—24 hours	441	<16	D—24 hours.

D = Died. S = Survived.

There is a puzzling discrepancy in this respect with the work of Todd (1933), who was able in recorded experiments to work with rabbits whose sera were practically devoid of antibody to serum-free hæmolysin. This is the more surprising because our serum-free hæmolysin, although it differed from Todd's in its greater stability, closely resembled it in its antigenic activity and in initiating hæmolysis more rapidly, and showing a sharper end-point in a hæmolytic titration than does a serum hæmolysin. In view, therefore, of the irregularities recorded with the control rabbits in this second series, a third series was immunized, but in this instance chinchilla rabbits were avoided, and a preliminary selection of rabbits with a low or relatively low antihæmolytic serum titre was made as in the earlier experiments of McLeod and McNee (1913) on the toxic effect of serum hæmolysin.

A preliminary rough titration of the antihæmolytic titre of the sera of 145 rabbits was made, and as a result 48 rabbits of which the sera did not contain more than 100 antihæmolytic units per c.c. were selected. A second and more careful titration of the antihæmolytic content of the sera of these rabbits was then made. On this occasion the hæmolysin used was more

readily neutralized and the titres read higher. The range of titre observed was from <12 to 300.

These rabbits were now split into four groups in such a way that animals with sera of very low, low and moderate antihæmolytic activity were evenly distributed in each group.

Group A was then immunized with serum-free hæmolysin.
,, B ,, ,, horse-serum hæmolysin.
,, C ,, ,, rabbit-serum hæmolysin.
,, D served as control and received no injection.

The hæmolytic filtrates administered to rabbits in Group A were obtained from 12–14 hours' cultures in the medium described on p. 245. The filter used was a Maassen candle. The M.H.D. of the various filtrates ranged from 0·2 c.c. to 0·08 c.c. Doses were given at weekly intervals subcutaneously, and were gradually increased from 2·5 c.c. to 10 c.c. In all just over 100 c.c. of filtrate were administered in the course of 13 weeks.

The rabbits in Group B received filtrate from 20 per cent. horse-serum broth cultures. The serum was obtained from Messrs. Burroughs Wellcome & Co.—sterile and free from antiseptic—and was heated for one hour at 56° C. before use. The broth was from a fresh meat extract and Parke Davis & Co. peptone, prepared according to H. D. Wright's method. The period of culture was 9½ to 13 hours, and other details are as for Group A, except that the hæmolytic titre of filtrates was higher—0·01 c.c. to 0·025 c.c.—and that the total administered was about 90 c.c.

The rabbits in Group C received the filtrates of cultures in 20 per cent. rabbit serum broth. In this case freshly-drawn serum was used after inactivation at 56° C. for 1 hour. The other details resemble those of the last series except in respect that more difficulty was experienced in getting the hæmolysin to pass through the filter, with the result that some samples were discarded as too weak and the total amount injected fell to 70 c.c., because on one or two weeks the injection was omitted. The M.H.D. also varied more widely, *i. e.* from 0·0125 c.c. to >0·1. It was noted, however, that even when 0·1 gave only almost complete hæmolysis, there would still be considerable hæmolysis with 0·01 c.c. In this respect there was a definite contrast with the serum-free hæmolysin, with which the end-point was much sharper.

All filtrates used for titrations of antihæmolytic activity and for immunization of animals were tested for their sterility by incubating 3 c.c.–5 c.c. in 10 c.c. of nutrient broth. All proved sterile.

After 14 weeks the series of immunizing injections was terminated, and 11 days later the antihæmolytic titres of the rabbits' sera from all three series and also from the controls was carried out. In determining the antihæmolytic titre of the sera a series of graded quantities of serum—from 0·2 down to 0·0001—was used. These quantities were mixed with 2½ M.H.D. hæmolytic filtrate and incubated for one hour before blood was added. The hæmolysin used was serum-free hæmolysin and the M.H.D. usually lay between 0·025 and 0·06. The reading was made after 2 hours' incubation and 2 or 3 hours at room temperature, and the smallest amount of serum which caused almost complete inhibition of hæmolysis was taken as index of the antihæmolytic activity of the serum. The figures recorded in the tables are obtained by

multiplying the reciprocals of such factors by the numbers of M.H.D. neutralized, and therefore correspond approximately to the number of anti-hæmolytic units per c.c. of serum.

Finally, three weeks after the last immunizing injections all the animals were given a test dose of 8–10 c.c. of filtrate intravenously. These injections were carried out in batches of 12, including 3 from each group, and all were completed within a week. The results of preliminary and subsequent titrations of antihæmolysin together with the toxic effects of the final injections are recorded for the several groups in the Tables VII, VIII, IX, X and XI.

TABLE VII.—*Controls.*

Rabbit No.	Titrations of antihæmolysin. First titration.	Second titration.	Test of lethal effect of toxin.
516	300	180	D—24 hours.
606	50	120	,,
609	210	450	D—30 hours.
622	200	180	D—2 days.
640	100	270	D—24 hours.
651	25	130	D—2 days.
Average titre for 12 rabbits*	103	207	

D = Died.

* The detail is only given for six.

TABLE VIII.—*Immunization with Serum-free Hœmolysin.*

Rabbit No.	Titrations of antihæmolysin. Before immunization.	After immunization.	Test of lethal effect of toxin.
514	210	3600	S
515	100	2250	S
527	210	4500	S
533	300	2000	S
538	50	4400	S
542	50	Died before titration	..
551	210	10500	S
552	210	1700	S
553	20	6750	S
561	25	7000	S
564	25	1575	S
589	<12	1575	S
Average titre	118	4168	

S = Survived.

TABLE IX.—*Immunization with Rabbit-Serum Hæmolysin.*

Rabbit No.	Titrations of antihæmolysin.		Test of lethal effect of toxin.
	Before immunization.	After immunization.	
534	100	2250	S
535	50	1150	D—4 days.
565	80	2400	S
569	80	1300	S
572	210	1100	S
577	50	2200	D within 15 min.
600	50	2250	S
605	210	3375	S
645	50	810	S
648	25	270	S
649	<12	675	D—12 days.
650	25	270	S
Average titre	78	1504	

D = Died. S = Survived.

TABLE X.—*Immunization with Horse-Serum Hæmolysin.*

Rabbit No.	Titrations of antihæmolysin.		Test of lethal effect of toxin.
	Before immunization.	After immunization.	
547	100	180	S
548	210	450	S
550	100	180	S
556	200	200	S
567	200	190	S
581	50	1050	D—24 hours.
583	50	45	S
590	<12	270	S
599	20	60	S
632	210	260	S
638	25	<20	D—24 hours.
641	25	<20	S
Average titre	100	230	

D = Died. S = Survived.

TABLE XI.

Method of immunization.	Number of rabbits.	Average anti-hæmolytic titres.	Lethal effect of I.V. hæmolysin.
Streptolysin from glucose buffered broth . .	11	4168	0/11 died.
Streptolysin from 20 per cent. horse-serum broth .	12	230	2/12 ,,
Hæmolysin from 20 per cent. rabbit-serum broth	12	1504	3/12 ,,
Control—no streptococcal filtrate . . .	12	207	12/12 ,,

These results show very clearly the value of a preliminary selection of rabbits with a view to eliminating those with a natural or acquired resistance to streptolysin. There is a uniform rise of antihæmolytic titre in the sera of the A group, as the result of the series of injections, and with this rise of antihæmolytic titre there is an associated resistance to the lethal effect of this type of toxin preparation, shown in 100 per cent. protection of these animals as compared with the controls.

The B group rabbits confirm the result obtained in the earlier series of experiments, that is, there is no stimulation of production of antihæmolysin to serum-free hæmolysin by injection of serum hæmolysin when horse-serum is used for preparation of the medium as in most of the earlier work on this subject.

It is clear, however, that a considerable degree of immunity to the lethal effect of the toxin may be developed independently of the production of antihæmolysin. It would, therefore, seem probable that hæmolysin and lethal toxin, although closely associated, are not identical. This apart, the results with rabbit-serum broth hæmolysin show a surprising discrepancy with those obtained with horse-serum hæmolysin in the respect that considerable antibody formation is provoked by the injection of hæmolytic filtrates from the former.

DISCUSSION.

It cannot be seriously claimed that recent work on streptococcal toxin has tended to simplify this problem. The work of Czarnetsky, Morgan and Mudd (1938) and Czarnetsky and Morgan (1938) indicates the existence of an ether-soluble, acetone-insoluble hæmolysin, which can be extracted from streptococci, is both a leucocidin and a general toxin, which has marked heat stability, acts as a haptene, but does not function as an antigen, and yields on decomposition by boiling with alkali a crystalline product with all the qualities of the original, except that it has been stripped of its nitrogen and phosphorus and no longer acts as a haptene.

It is desirable, so far as possible, to correlate the results recorded in this paper with those just cited on the one hand, and with those of Todd on the other. Todd (1938), in his most recent paper on this subject, suggests that all streptococcal hæmolysins as yet described consist of two hæmolytic elements in varying proportions—an oxygen-sensitive and antigenic streptolysin O, and

an S streptolysin less sensitive to oxygen, and incapable of stimulating antibody formation that can be demonstrated in a direct experiment.

If this is so, Todd's (1933) earlier contention that S streptolysin, although incapable of neutralization by the serum of an animal to which it has been injected, yet functions as an antigen stimulating antibody production to O streptolysin, falls to the ground. The increased antibody to O streptolysin which he demonstrates in animals injected with S streptolysin would, in view of his later work, be most readily explained by the O streptolysin content of his serum streptolysin filtrates. To reconcile our work with that of Todd, however, it will be necessary to suppose either (*a*) that some fundamental factor is being ignored in his explanation of the phenomena observed, or (*b*) that he is wrong in supposing that all hæmolytic filtrates contain both S and O streptolysins, and that the horse-serum broth cultures used in the experiments described in this paper contain only S streptolysin which is non-antigenic, whereas the rabbit serum broth cultures contain both S and O streptolysins.

Facts, when fully elucidated, are often more surprising than any flight of speculation, but on *a priori* grounds the likelihood that one micro-organism will produce two distinct varieties of hæmolysin does not seem great. We are inclined, therefore, to wonder whether there is not some underlying factor which will reconcile all these conflicting observations on streptococcal hæmolysin; both those of Todd and our own, and still more that of Czarnetsky, Morgan and Mudd on the thermostable nature of their extracted hæmolysin, and all the earlier work which emphasizes with such unanimity the thermolabile character of streptolysin. The contrast between the antigenic activities of streptolysins prepared from rabbit- and horse-serum broth cultures respectively which is recorded in this paper brings to mind the contrast observed by Avery and Tillett (1929) between antipneumococcal serums from the rabbit, which can sensitize guinea-pigs to a subsequent injection of homologous pneumococcal polysaccharide, and antipneumococcus serum from the horse which cannot. This result was confirmed by Brown (1933). Again, Neill, Sugg and Richardson (1932) have shown that antidiphtheria serum from man, rabbit and guinea-pig can sensitize guinea-pigs to subsequent injection of diphtheria toxin, whereas horse-serum cannot. This work has been confirmed and extended by Hartley (1938), and also in part corroborated by Schafer (1938). Hartley showed that ox and goat sera were similar to horse-serum and contrasted with rabbit, guinea-pig and human sera in this respect.

It would seem not unlikely that streptolysin is a substance readily forming complexes with other organic molecules, which may modify both its powers of resistance to heat or oxidation and its capacity for functioning as an antigen.

These are, of course, little more than speculations, but if anything can be worked out on such lines to deliver the subject from its present excessive complexity, it will be a welcome advance.

Antitoxin Content to Lethal Toxin in the Sera of Immunized Animals.

Pulvertaft (1929) has shown that the streptococcal toxin with which he worked was neutralized by antiscarlatinal horse-serum. Both with a view to comparing our toxin with his in this respect, and in order to control the possible

antitoxic activity of the sera of immunized rabbits, a series of experiments was made in which protection of rabbits was attempted after the injection of doses of toxin likely to be lethal to most rabbits.

The results of these experiments are submitted in Table XII. This is a small series of experiments in which 75 per cent. of the controls and 60 per cent. of animals treated with the antitoxin under investigation died, but none of those receiving antiscarlatinal serum in equal amount. It is clear, therefore, that antiscarlatinal serum has definite protective qualities against this toxin, and that the serum of the immunized rabbit is much less effective, if effective at all. The failure of the latter to protect may be due to the fact that serum was only taken for this purpose when a considerable period after the termination of the series of immunizing injections had elapsed. In any case the definite protection conveyed by antiscarlatinal serum is a point of considerable interest, since it indicates that such serum contains antitoxin to toxin prepared from streptococci in a variety of ways, and hence increases the experimental justification for its use in streptococcal infections of man.

TABLE XII.—*Toxin-Neutralizing Powers of Various Antisera.*

Dose of toxin.	Route.	Treatment.	Number of animals injected.	Number which died.
10 c.c.	I.V.	5 c.c. normal horse-serum	3	3
,,	,,	5 c.c. antidiphtheria serum (B.W. & Co.)	3	2
,,	,,	5 c.c. homologous rabbit-serum	5	3
,,	,,	5 c.c. antiscarlatinal serum (B.W. & Co.)	5	None.
,,	,,	1 c.c. antiscarlatinal serum (B.W. & Co.)	2	1 late.
,,	,,	None	6	4

SUMMARY.

An extract of meat prepared at temperatures not exceeding 75° C. was used as a basis in preparing Hewitt and Todd's glucose buffered broth medium.

Certain strains of streptococcus when grown in this medium produce cultures from which actively hæmolytic filtrates can be obtained.

The streptolysin obtained in this way resembles that described by Todd in respect of its rapid action on blood and sharp end-point in titrations of its activity as well as in being definitely antigenic. It differs, however, in much greater stability. No reduction of the filtrate is necessary, and the activity often remains unimpaired after two days' sojourn in the ice-chest in a wide tube plugged with cotton-wool. These filtrates exhibit a definite lethal action, the most active observed having an M.L.D. of 0·5 to 1 c.c. for the rabbit by subcutaneous or intramuscular injections.

There is a wide variation in the antihæmolysin content of rabbits' sera ("normal rabbits") to this hæmolysin, and the resistance of the animals to

lethal toxin runs roughly parallel to the antihæmolysin content of the sera. After 9 or 10 weeks' immunization with this filtrate a marked rise in the antihæmolytic titre of the serum is observed, and with this is associated an increased resistance to the lethal action of the filtrate.

No increase in antihæmolytic activity of the serum for the type of streptolysin described was observed after injection with serum streptolysin obtained from horse-serum broth, whereas a considerable rise followed injection of serum hæmolysin from rabbit-serum broth.

The discrepancy between the immunity developed to hæmolysin and to lethal toxin in the animals injected with horse-serum broth hæmolysin make it difficult to believe that hæmolysin and lethal toxin are identical.

The discrepancies between these observations and those of Todd on the one hand and between both of these and the results of Czarnetzky, Morgan and Mudd, who extracted thermostable toxin from streptococci on the other must be elucidated before further progress in this rather complicated field will be possible. The lethal toxin described is neutralized by antiscarlatinal horse-serum, but very little antitoxin has been demonstrated in the serum of immunized rabbits.

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PSEUDO-LYMPHOCYTIC CHORIOMENINGITIS.

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In 1934 Armstrong and Lillie in America first isolated the virus of lymphocytic choriomeningitis from the brain of a rhesus monkey, which had been inoculated intracerebrally with passage material originally obtained from the cerebro-spinal fluid of a patient supposedly suffering from St. Louis encephalitis. Since then various observers in the U.S.A., England, France and Japan have isolated, either from patients suffering from meningitis or from stocks of apparently normal mice (Rivers and Scott, 1935; Traub, 1935; Findlay *et al.*, 1936; Lépine and Sautter, 1936; and Kasahara *et al.*, 1939), viruses which, though varying slightly in their pathogenicity for different laboratory animals, are identical, or very closely related, as shown by the immunological reactions and the pathological changes produced.

During the past few years, thanks to the kindness of numerous clinicians, it has been possible to examine cerebro-spinal fluid, blood and other materials from cases which on clinical grounds were diagnosed as benign aseptic lymphocytic meningitis. These examinations have resulted in the isolation of several strains of a virus which does not differ from that of lymphocytic choriomeningitis. These strains will be dealt with in a further communication. In addition, on two occasions at an interval of some 8 months, what appears to be another virus has been obtained. This virus, which in certain respects resembles that of ordinary lymphocytic choriomeningitis, we have termed "pseudo-lymphocytic choriomeningitis". The actual source of the virus, whether from human or animal material, requires fuller discussion, but for the sake of completeness notes are included on the clinical histories of the two patients from whom the virus is presumed to have been isolated.

SOURCE OF THE VIRUS.

Case 1.

C. D—, a 14-year-old male. Illness began with severe frontal headache, drowsiness, irritability and vomiting. The headache and drowsiness continued for 7 days, at the end of which time he was admitted to hospital. On admission there was some rigidity of the neck but no head-retraction, and the presence of Kernig's sign was doubtful. The plantar reflexes were flexor and the abdominal reflexes absent. There were no other abnormal physical

signs. He was treated by repeated lumbar puncture and slowly improved till the 40th day of illness, when he was discharged completely recovered.

Laboratory results.

The spinal fluid on admission was under slightly increased pressure. Protein 60 mg. per cent., chlorides 740 mg. per cent., cells 14 per c.mm., all lymphocytes. The following day, the 8th of illness, only 6 cells, all lymphocytes, were seen per c.mm., and this was the material received at this laboratory. The cerebro-spinal fluid was inoculated in 0·03 c.c. amounts intracerebrally into 4 mice. One mouse was dead on the 6th day; one was sick on the 8th day, sitting quietly in the corner of the box with roughened fur. It was killed, the brain removed, cultured and found to be bacteriologically sterile, ground in normal saline and made up as a 20 per cent. suspension. This suspension was inoculated intracerebrally into 6 mice, but none became sick, and later all succumbed to re-inoculation with the virus under discussion. The third of the original mice was sick on the 9th day with symptoms typical of lymphocytic choriomeningitis in the mouse. This mouse was killed and the brain removed and treated in the manner already described. The six mice inoculated with the brain suspension of this mouse were all dead or sick on the 9th day with symptoms typical of lymphocytic choriomeningitis. A third passage from one of these mice resulted in death or sickness in 4 or 5 days with the same symptoms, and this interval from inoculation to death has continued now for over 60 passages. The mice inoculated with the patient's blood taken the same day as the cerebro-spinal fluid died on the 10th, 13th, 13th and 18th days with the same symptoms and pathological changes as those seen in mice inoculated with the cerebro-spinal fluid.

The cerebro-spinal fluid and blood were inoculated intracerebrally and intraperitoneally into 2 guinea-pigs with negative results.

A rhesus monkey inoculated intracerebrally with 1·0 c.c. of the cerebro-spinal fluid remained well; its immunity was not tested.

Case 2.

T. S—, a 7½-year-old female, was admitted to hospital with a history of frontal headache for 5 days and occasional vomiting and squint for 1 day. On admission her temperature was 101·4° F. On examination there was some paresis of the left 6th and doubtful weakness of the left 7th cranial nerves; nystagmus on looking to the left. There was slight stiffness of the neck muscles, but otherwise the whole of the central nervous system appeared normal. There was no swelling of the optic discs. The temperature gradually returned to normal, though the patient's condition remained unchanged. On the 10th day slight stiffness of the neck was still present and a definite bilateral Kernig's sign. The nystagmus and neck stiffness disappeared in the next few days, but there was still occasional vomiting. The patient was allowed up one month after admission, and was discharged at the end of two more weeks completely recovered, except for paresis of the left 6th cranial nerve. Fifty-nine weeks later some weakness of the left external rectus muscle was still present, but there was steady recovery.

Laboratory results.

A lumbar puncture on the 6th day of illness yielded clear cerebro-spinal fluid at 110 mm. pressure. Protein ·025 per cent., globulin normal, chlorides 737 mg. per cent; 40 white cells, chiefly lymphocytes, per c.mm. Cerebro-spinal fluid was again removed on the 13th day with no change in protein, globulin or chlorides and only 10 cells, all lymphocytes, per c.mm. This fluid was the material received at this laboratory.

Twelve mice were inoculated intracerebrally with 0·03 c.c. One of the mice died on the 22nd day following inoculation. The brain after removal was found to be bacteriologically sterile and a suspension was prepared in the usual manner. Mice inoculated intracerebrally with this material became sick on the 5th or 6th day, with symptoms typical of lymphocytic choriomeningitis. Further intracerebral passage from these mice resulted in death or sickness in 4 or 5 days with the same clinical picture as seen with the virus obtained from Case I. The same clinical picture has continued for more than 50 passages.

A rhesus monkey inoculated intracerebrally with 1·0 c.c. of the 3rd passage mouse brain suspension remained well, but was later immune to intracerebral inoculation with mouse brain suspension of a later passage.

THE EXPERIMENTAL DISEASE.

The disease has been experimentally studied in mice, monkeys and guinea-pigs.

Mice.

The two strains produce an identical clinical and pathological picture in mice, infection following intracerebral, intranasal, intraperitoneal and subcutaneous inoculation. The incubation period after intracerebral inoculation, which has been the routine method of maintaining the virus, is 4 to 5 days, except with high dilutions such as 10^{-5} or 10^{-6}, when symptoms may not appear till the 6th to 8th day. The clinical symptoms in mice are identical with those of true lymphocytic choriomeningitis, except for the latter disease's longer incubation period of 6–8 days with dilutions of 10^{-1} and up to 14 days for 10^{-5} and 10^{-6}. Shortly before death the mice remain quietly hunched up in a corner of the box, occasionally with a slight coarse spontaneous tremor, while the fur is roughened. If the mice are stimulated in any way, as by shaking the box, being touched or spun by the tail, they exhibit a series of clonic movements, ending in a tonic convulsion with rigid extension of the hind legs and flexion of the forelegs. The mice may recover from this spasm and survive for a few hours or they may die instantly. Intraperitoneal or subcutaneous inoculation of 0·2 c.c. of a dilution of infected mouse brain 10^{-3} usually results in sickness or death in from 6 to 10 days, with symptoms similar to those following intracerebral injection. Following intranasal instillation of 0·03 c.c. of a suspension of virus in dilutions up to 10^{-3}, in etherized mice, 50–75 per cent. of the animals on about the 6th day develop roughening of the fur, sluggish movements and a sticky exudate which

glues the eyelids together, but no neurological symptoms such as the spasms already described. On post-mortem examination these intranasally inoculated mice show complete consolidation of one or more lobes of the lungs. An occasional mouse, however, shows nervous symptoms only, and on pathological examination no macroscopic lesions are seen in the lungs, although microscopic lesions may be present.

At death, both after intracerebral inoculation and intranasal instillation, virus is found in moderate amounts in the blood, brain, lung, spleen and liver.

TABLE I.—*Susceptibility to Inoculation with the Virus of Pseudo-lymphocytic Choriomeningitis.*

Species.	Infection with death. Strain.		Survival of virus. Strain.	
	T.S.	C.D.	T.S.	C.D.
Guinea-pig :				
i.c.	++	—	..	—
i.n.	++	—	..	—
i.p.	++	—	..	—
s.c.	++	—	..	—
Macacus rhesus :				
i.c.	++	+	..	..
s.c.	—	—	—	—
i.p.	—	—	—	—
Dog :				
i.c.	—	—	—	—
Ferret :				
i.c.	—	—	—	—
i.n.	—	—	—	—
i.p.	—	—	—	—
Rat :				
i.c.	—	—	9 days	9 days
Rabbit :				
i.c.	—	—	—	—
i.p.	—	—	—	—
s.c.	—	—	—	—
Hedgehog :				
i.c.	—	—	—	—
Hen :				
i.c.	—	—	10 days	10 days
Pigeon :				
i.c.	—	—	—	—
Canary :				
i.c.	—	—	—	—

i.c. = intracerebral. i.n. = intranasal. i.p. = intraperitoneal. s.c. = subcutaneous. ++ = invariably fatal. + = mortality 50 per cent.

When undiluted urine from moribund mice is injected intracerebrally, about 25 per cent. of the mice develop symptoms of the disease, and approximately 90 per cent. of the remainder, though not apparently sick, are immune to intracerebral inoculation of the virus two weeks later. There thus appears to be considerably less virus present in the urine than is the case in mice infected with lymphocytic choriomeningitis. Occasionally after several weeks' contact with infected mice, normal ones became sick or were subsequently found to have developed immunity on intracerebral or intranasal inoculation. No infection or immunity was obtained by oral administration.

Monkeys.

In the rhesus monkey there is a difference in susceptibility to the two strains. Intracerebral inoculation of six animals with 0·5 c.c. of a 10 per cent. suspension of mouse brain of the T.S. strain caused death in every case. The usual sequence was a gradual fall of temperature to 99·6° or 99° F. on the 5th to 8th day after inoculation, with development of paresis and clonic spasm of the hind legs and, occasionally, grinding of the teeth, followed by complete collapse and death in about 24 hours.

Virus was always present at death in the brain, twice in the spleen, but only once in the blood and never in the other organs. In contrast to infection with lymphocytic choriomeningitis, at no time was there a rise in temperature after inoculation with the T.S. strain.

When the C.D. strain was used, only 3 of the 6 inoculated monkeys died. The symptoms and temperature changes were as a rule similar to those infected with the T.S. strain, but in two monkeys there was a rise of temperature to 103·8–104·4° F., commencing on the 5th to 7th days and lasting a day or two before dropping to subnormal level. Death occurred in the three monkeys 7, 7 and 9 days after inoculation.

On subcutaneous and intraperitoneal inoculation, neither strain produces any abnormal signs or symptoms.

Guinea-pigs.

The T.S. strain was again the more virulent. Intracerebral inoculation of a guinea-pig with 0·2 c.c. of a 10 per cent. mouse brain suspension of the 30th mouse passage resulted in elevation of the temperature to 104·7° F. on the 9th day, rising to 105·8° F. on the 13th day and falling to 99·8° F. on the 15th day. The guinea-pig was then killed, since it had become thin and weak, the appearance being very similar to that caused by lymphocytic chorio-meningitis. A 20 per cent. suspension of the brain of this guinea-pig inoculated intracerebrally, intraperitoneally, subcutaneously and intranasally into guinea-pigs caused in each instance a similar clinical picture. At death a moderate amount of virus was present in the blood, lungs, liver, spleen and kidney and a small amount in the urine.

In contrast to the above results, it has been impossible to induce symptoms in guinea-pigs with the C.D. strain of virus, while those inoculated with the C.D. virus all succumb later when injected with the T.S. strain.

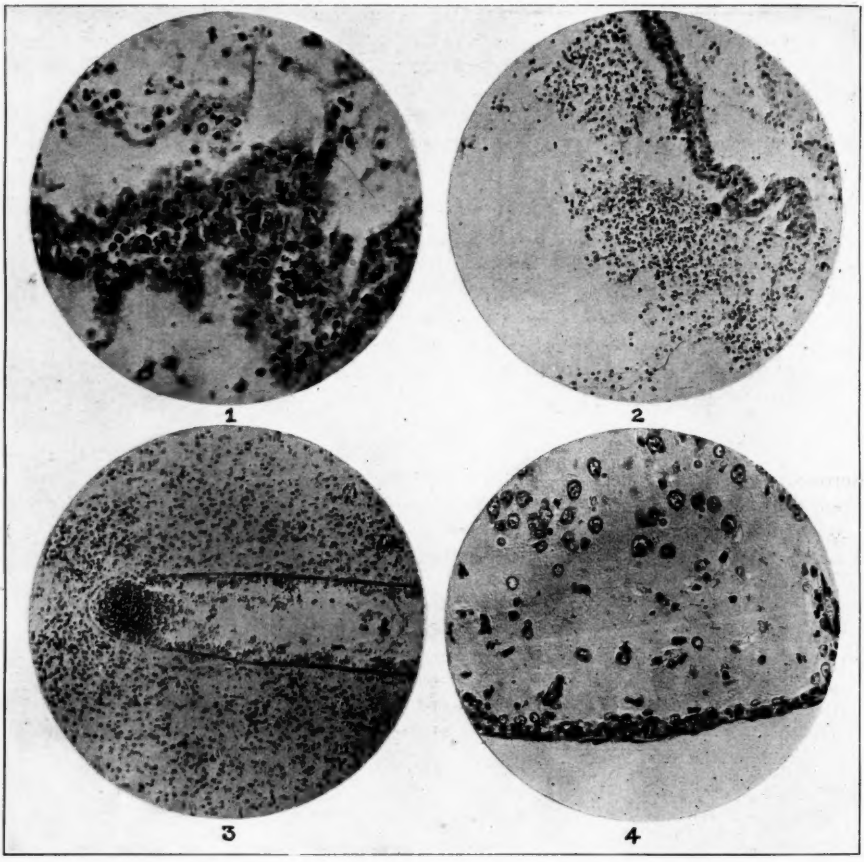

FIG. 1.—Brain of mouse, showing involvement of choroid plexus and ventricles. × 100.

FIG. 2.—Brain of mouse, showing cells infiltrating choroid plexus. × 200.

FIG. 3.—Brain of mouse, showing infiltration of ventricle and parenchymal tissue. × 70.

FIG. 4.—Brain of monkey, showing cells infiltrating meninges. × 225.

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Other animals.

Following intracerebral inoculation with 20 per cent. infected mouse brain suspension, the virus survived 9 days in the brain of the rat and 10 days in the brain of the hen, but no symptoms were produced and continued passage failed. In the dog, ferret, rabbit, hedgehog, pigeon and canary there was no evidence of the presence of virus in the brain or blood on the 3rd day following intracerebral inoculation. No rise of temperature and no local changes in lymph-nodes or skin followed subcutaneous inoculation.

Pathology.

Very frequently there is little to be seen, either macroscopically or microscopically, in the organs of animals moribund or dead of the disease. When changes are present, the pathological picture is similar to that met with in true lymphocytic choriomeningitis, except that the lesions are less widespread, while on microscopical examination the cellular exudate contains a relatively larger percentage of polymorphonuclear leucocytes than in the latter disease.

Mice.

In mice dying after intracerebral inoculation of virus there are no macroscopical changes in any organ. After intranasal instillation, more than 50 per cent. of the mice die and show complete consolidation of one or more lobes in the lungs accompanied by a glairy pleural exudate.

Histological examination often shows surprisingly little pathological change. In the brain there is some infiltration of the meninges and choroid plexus, with mononuclear and polymorphonuclear leucocytes and occasional perivascular cuffing with the same cells. The cells of the choroid plexus appear slightly swollen (Fig. 2) and the ventricles contain masses of cells (Figs. 1 and 3). The lungs show scattered areas of round-cell infiltration, chiefly in the region of the blood-vessels, and small areas of œdema. No changes have been detectable in any other organ.

Monkeys.

Here there are no gross changes to be seen, and the microscopical lesions, which are confined to the brain, meninges (Fig. 4) and lung, are the same as seen in the mouse.

Guinea-pigs.

When these animals are infected with the T.S. strain the same lesions in the form of cell infiltration of brain, meninges and lung are seen.

CHARACTERS OF THE VIRUS.

The agent producing the above disease appears to belong to the class of filterable viruses, since no bacteria have been cultivated on the more commonly employed aërobic and anaërobic media, or on such special media as that used for growing pleuropneumonia-like organisms. On the other hand, both

strains of the virus pass through a Berkefeld V and occasionally through a Seitz E.K. filter, though never through a Berkefeld N candle. Both strains, however, as shown by the ability of the filtrates to kill or immunize mice on intracerebral inoculation, are able to pass through gradocol membranes of an average pore diameter of 320 mμ., but not of 300 mμ. The agents therefore do not exceed 150–225 mμ. in size.

A small amount of virus is still present in the supernatant fluid after centrifugation of an infected mouse brain suspension in saline at 8000 r.p.m. for 1 hour, but complete sedimentation occurs after 1 hour at 12,000 r.p.m. in a high-speed Baskerville centrifuge.

The virus survives for at least one month when kept at 4° C., and for a year either in 50 per cent. glycerine in saline or after freezing and drying.

The virus is killed by boiling for 5 minutes and by heating at 56° C. for 30 minutes. It survives exposure to 45° C. for 30 minutes. Complete inactivation occurs after 48 hours' contact with a final concentration of 0·05 per cent. formalin at 4° C., but 0·25 per cent. phenol takes more than 40 days to kill the virus.

The virus is readily maintained on the chorio-allantoic membrane of the developing chick embryo, but has not up to this point been grown successfully in serum Tyrode medium containing either chick or mouse embryo tissue. When inoculated *in vivo* the virus multiplies readily in the cells of mouse carcinoma 63 and sarcoma 37 S.

IMMUNITY.

Mice which recover after intracerebral or intranasal inoculation are usually resistant to reinoculation by either route, while mice immunized with sub-lethal doses or recovered from the disease induced by one strain are subsequently immune to both strains of the virus.

Monkeys which recover from intracerebral inoculation with the C.D. strain are subsequently immune to inoculation with the more virulent T.S. strain by the same route.

Attempts to study serological immunity in animals using a mouse protection test similar to that employed for detecting virucidal antibodies in lymphocytic choriomeningitis sera (Armstrong and Wooley, 1935) have so far not been successful. Sera from rabbits receiving repeated intraperitoneal injections of virus fail to protect regularly when serum-virus mixtures are injected intracerebrally or intraperitoneally in mice. This question is being studied further, and it is hoped to report the results on the production of immune bodies in the various animals inoculated at a later date.

The serum from the patient C. D—, taken 30 days after the onset of the disease, when tested in mice gave protection against virus in a dilution less than 1 : 10, whereas no other serum used as control, including that of a supposedly hyperimmune rabbit, neutralized virus in a dilution of less than 1 : 100. Serum taken from the same patient 7 months after the onset of illness gave protection only when virus was diluted 1 : 10,000 as did other normal sera, whereas a hyper-immune rabbit serum neutralized virus in a dilution of 1 : 255.

Due to unavoidable difficulties, it was not possible to obtain serum from the patient T. S— until one year after the onset of her illness, and there were then no demonstrable neutralizing antibodies present.

DISCUSSION.

Following the inoculation into the brains of mice of bacteriologically sterile cerebro-spinal fluid from two patients suffering from a disease which clinically suggested benign aseptic lymphocytic meningitis, two strains of what appears to be the same virus have been isolated. The results of tests for neutralizing antibody in the patients' sera suggest that at least one of the strains, C.D., may be of human origin. If the virus is of murine origin it is strange that it has not previously been isolated in view of the large numbers of mice now employed in laboratories. There is no history of contact with mice, nor any suggestion of the source of the infection. The agents were isolated at an interval of about 8 months in a small number of the mice inoculated with the original material in each case. As in the case of various strains of lymphocytic choriomeningitis virus, differences have been detected in the susceptibility of monkeys and guinea-pigs.

Though the laboratory animals susceptible are the same and the clinical and pathological pictures are very similar to those of lymphocytic choriomeningitis, there is no immunological relationship with this virus. Neither mice immune to lymphocytic choriomeningitis virus (Table II) nor hyperimmune sera prepared in the rabbit against it (Table III) have any protective power against the new virus, and *vice versa*.

Preliminary filtration experiments suggest the size of the new virus is between 150–225 mμ., while McNair Scott and Elford (1939) suggest 40–60 mμ. for the size of lymphocytic choriomeningitis virus.

Similarly no cross-immunity could be demonstrated with the virus of St. Louis encephalitis, or with the virus causing acute meningitis and pneumonitis isolated by Francis (1938), or with the neurotropic strain of influenza virus recently produced in the mouse by Stuart-Harris (1939) (Table II). No lesions could be produced in the ferret with the two strains of our meningitis virus. The clinical and pathological picture is quite different to that of the mouse encephalomyelitis described in America by Theiler (1934)—a disease which up to the present has not been met with in this country.

The nomenclature of viruses arising spontaneously in the mouse or isolated following intracerebral inoculation of mice with various material is now becoming rather involved, and although open to certain objections, it is felt that this new condition is best described as pseudo-lymphocytic choriomeningitis.

CONCLUSIONS.

Two strains of what appears to be the same virus have been isolated from mice following intracerebral inoculation of cerebro-spinal fluid from patients with symptoms of benign aseptic lymphocytic meningitis.

This virus produces a clinical and pathological picture in mice, guinea-pigs and rhesus monkeys very similar to that of lymphocytic choriomeningitis,

TABLE II.—*Intracerebral Inoculation of Viruses into Immune Mice: Cross-immunity Tests.*

Virus inoculated.	Mice immune to						Normal.
	C.D.*	T.S.*	Lymphocytic choriomeningitis.	St. Louis encephalitis.	Meningitis and pneumonitis.	Neurotropic influenza.	
C.D.	S, S, S, S, S, S	S, S, S, S, S, S	4, 4, 4, 4, 5, 5	4, 4, 4, 4, 4, 5	4, 4, 4, 4, 4, 4	4, 4, 4, 4, 5, 5	4, 4, 4, 4, 5, 5
T.S.	S, S, S, S, S, S	S, S, S, S, S, S	4, 4, 4, 4, 4, 5	4, 4, 4, 4, 5, 5	4, 4, 4, 4, 4, 5	4, 4, 4, 4, 4, 5	4, 4, 4, 4, 4, 4
Lymphocytic choriomeningitis	6, 6, 7, 7, 7, 7	6, 7, 7, 7, 7, 7	S, S, S, S, S, S	..	..	..	6, 6, 7, 7, 7, 7
St. Louis encephalitis.	6, 7, 7, 7, 8, 8	6, 7, 7, 7, 8, 8	..	S, S, S, S, S, S	..	..	6, 6, 6, 6, 7, 7
Meningitis and pneumonitis (Francis)	4, 6, 6, 6, 6, 6	4, 5, 6, 6, 6, 6	..	..	S, S, S, S, S, S	..	3, 4, 6, 6, 6, 6
Neurotropic influenza.	4, 4, 4, 5, 5, 5	4, 4, 4, 4, 5, 5	..	..	..	S, S, S, S, S, S	3, 3, 4, 4, 4, 5

Virus = 0·025 c.c. of 10 per cent. suspension of mouse brain in saline inoculated intracerebrally.
The numbers indicate the days from inoculation to death.
S = survival.

* C.D. and T.S. = two strains of pseudo-lymphocytic choriomeningitis.

TABLE III.—*Neutralization Test with Rabbit Sera.*

Virus.	Serum. W.E.	T.S.	C.D.	Normal.
W.E. 10^{-2}	9, S, S, S, S, S	6, 6, 7, 7, 7, 7	6, 7, 7, 7, 7, 7	6, 6, 7, 7, 7, 7
10^{-3}	S, S, S, S, S, S	7, 7, 8, 8, 8, 8	7, 8, 8, 8, 8, 8	7, 7, 8, 8, 8, 8
10^{-4}	S, S, S, S, S, S	8, 8, 8, 8, 9, 10	8, 8, 8, 8, 9, 9	8, 8, 8, 8, 9, 9
*C.D. 10^{-2}	5, 5, 5, 5, 6, 6	5, 5, 6, 6, 6, S	5, 5, 6, 6, 6, S	5, 5, 5, 5, 5, 6
10^{-3}	5, 5, 5, 6, 6, 6	5, 6, 6, 6, S, S	5, 6, 6, 6, 6, S	5, 5, 5, 6, 6, 6
10^{-4}	6, 6, 6, 6, 6, 7	5, 6, 7, 7, S, S	6, 6, 6, 6, 7, S	5, 6, 6, 6, 6, 7
*T.S. 10^{-2}	5, 5, 5, 5, 5, 6	5, 5, 5, 6, 6, S	5, 5, 6, 6, 6, S	5, 5, 5, 5, 6, 6
10^{-3}	5, 5, 6, 6, 6, 6	5, 6, 6, 6, 6, 6	5, 6, 6, 6, 6, S	5, 5, 6, 6, 6, 6
10^{-4}	5, 6, 6, 6, 6, 7	5, 6, 7, 7, S, S	6, 6, 6, 6, S, S	5, 6, 6, 6, 6, 7

Virus.—10 per cent. suspension of mouse brain in saline centrifuged at 1000 r.p.m. for 5 minutes; 10-fold dilutions made of supernatant.

Serum.—From rabbits having received repeated intraperitoneal inoculations of living virus.

Technique.—0·1 c.c. virus mixed with 0·2 c.c. of serum and allowed to stand 2 hours at room temperature, then 0·025 c.c. of mixture inoculated intracerebrally into each of 6 mice.

The numbers indicate the days from inoculation to death.

S = survival.

W.E. = River's original strain of lymphocytic choriomeningitis.

* C.D. and T.S. = two strains of pseudo-lymphocytic choriomeningitis.

except that in mice the incubation period following intracerebral inoculation is only 4 to 5 days and the pathological lesions are less extensive.

The results of animal inoculation and immunological tests prove it to be unrelated to any previously described virus.

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CATAPHORESIS EXPERIMENTS WITH SENSITIZED ERYTHROCYTES.

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THE mechanism of cytolysis by the combined action of serum-complement and a "sensitizing" agent such as a specific immune body has in the past attracted much attention, and a voluminous literature has accumulated on this subject. Its importance from the point of view of immunological theory is undobuted. When Landsteiner and Jagič (1904) first showed that hæmolysis could be produced by colloidal silicic acid along with serum, and when later it was found that the active substance of the serum was complement and that the silicic acid, as it were, replaced immune body in the hæmolytic system (Landsteiner and Rock, 1912; Liebers, 1913; Nathan, 1913; Browning and Mackie, 1914 and 1925), it appeared that sensitization of erythrocytes and other cells by an immune body might be explained on a physico-chemical basis.

Recently Goldie (1938) has suggested that the phenomenon of hæmolysis by colloidal silicic acid and serum depends on natural hæmolysin in the serum, the colloid forming a complex with anticomplementary substances and allowing the complement to act on the sensitized cells. This explanation does not seem tenable, since in the original work on the subject the possible intervention of a natural hæmolysin was carefully excluded by using erythrocytes and serum from the same animal and, where heterologous erythrocytes and sera were tested, by preliminary "absorption" of the serum with the particular cells to remove any hæmolysin present (see Landsteiner and Rock, 1912; Browning and Mackie, 1925).

Mudd and Mudd (1926), by examining the behaviour of sensitized red cells at the interface between oil and saline solution were able to demonstrate a loss of the lipoid character of the surface. Reiner and Fischer (1929) found that tannic acid (in colloidal solution) brought about hæmolysis in the presence of unheated serum, whereas with heated serum (the complement being inactivated) no such effect occurred. They argued by analogy that a hæmolytic immune body acts as a "dehydrating" agent, converting the cells from a stable hydrophilic to an unstable hydrophobic colloid system. The hæmolytic reaction they described with tannic acid would appear to be analogous to hæmolysis by colloidal silicic acid along with complement. The physical change postulated by them would, according to the accepted theories of colloid chemistry, involve a reduction in the surface charge of the red cells, and would also bring about agglutination of the cells in the presence of salts.

In this connection it is noteworthy that both colloidal silicic acid and tannic acid have been shown to possess strong agglutinative properties towards red cells, while a hæmolytic antiserum may or may not possess a corresponding action.

In the course of the work recorded in this paper further observations were made on the hæmolytic action of colloidal silicic acid and tannic acid along with serum, and by cataphoresis experiments an endeavour was made to ascertain whether sensitization by hæmolytic antiserum, cobra venom and the two chemical substances which may replace these in a hæmolytic system was associated with an alteration in the electrical charge on the surface of the cells. Thus according to the view of Reiner and Fischer sensitization would be accompanied by a reduction of the charge, and this should be manifest by a slowing in the migration of the cells in an electric field.

METHODS.

Hæmolysis experiments.

Sheep red corpuscles (washed) were suspended in physiological saline solution to yield a 3 per cent. suspension. Graded amounts of the reagents to be investigated were added to 0·5 c.c. of the cell suspension, the mixture was allowed to stand for 15 minutes and varying amounts of guinea-pig complement (diluted 1 in 10 with saline) were added. The tubes were incubated for 3 to 4 hours at 37° C., and then allowed to stand overnight at room temperature, when the results were read. Controls containing the reagent but no complement, and complement but no reagent, were included in each set.

Colloidal silicic acid was prepared by heating 2 g. of ethyl silicate and 100 c.c. of water under reflux for 3 hours. The solution was filtered and stored. Immediately before use an appropriate volume of 10 per cent. sodium chloride solution was added to give a final concentration of 0·85 per cent. sodium chloride.

Tannic acid was dissolved in physiological saline solution to form a 1 per cent. solution.

The organic acid solutions were prepared by adding 1 or 2 c.c. of a 1 per cent. alcoholic solution of the acid to 9 or 8 c.c. of physiological saline.

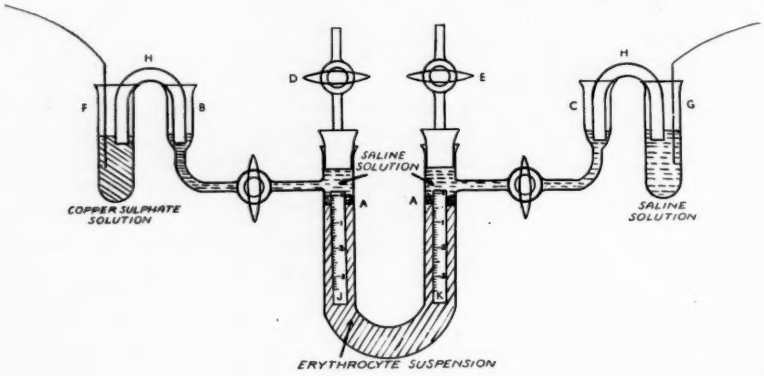
Complement.—In order to eliminate any effects due to the presence of natural immune body in the guinea-pig serum used as complement it was treated with an equal volume of washed red cells; the mixture was allowed to stand for 1 hour at 0° C. and the red cells were then removed by centrifuging.

Cataphoresis experiments.

A U-tube apparatus, with certain similarities to that described by Todd (1927), previously used in this laboratory for cataphoresis experiments with viruses was adapted for the present experiments. It may be noted that certain workers have preferred for such observations with erythrocytes a micro-electrophoretic cell (Moyer, 1936; Abramson, 1929) such as that described by Northrop and Kunitz (1925). This form of apparatus, however, does not lend itself to the direct simultaneous comparison of different suspensions of erythrocytes such as we have carried out with two or three U-tubes in series (*vide infra*). After preliminary trials with the U-tube apparatus consistent results in measuring the electrophoretic motility of erythrocytes were obtained and all our observations were made by this technique. Washed erythrocytes (sheep) were suspended in saline to yield a 10 per cent. suspension. It was found that lower concentrations of red cells gave rise to indefinite boundaries, making measurement difficult. The suspensions were placed in the U-tube (see figure) in a volume sufficient to fill the U to within about 0·5 cm. of the side tubes. Small plugs of cotton-wool (A) were placed just above the level of the cell suspension and below the side tubes in order to minimize disturbance of the boundaries during the course of the experiment. Saline was then allowed to flow into the space above the suspension from the reservoirs B and C, care being taken to exclude air-bubbles

and to avoid mixing the saline and suspension, thus maintaining a sharp boundary. Tap D was open and tap E closed whilst filling the left-hand space, and *vice versa* for the right-hand space. Usually two or three such tubes were filled with blood suspensions, one untreated as a control, and the others with cell suspensions to which had been added appropriate amounts of the substances under investigation. The tubes were then connected in series by means of short-limbed agar bridges (H), containing 4 per cent. of sodium chloride, dipping into the side tubes B and C and into electrode vessels F and G. (The figure shows the arrangement as applied to one tube only). The cathode consisted of a strip of platinum foil immersed in 5 per cent. copper sulphate solution, and the anode of platinum foil in 1 per cent. sodium chloride solution. Current was applied from the mains at 230 V. and measured by means of a milliammeter in series with the cells. The positions of the boundaries of the cell suspensions were recorded before and after the experiment by means of the millimetre scales J and K on the limbs of the tubes. The difference in level between the boundaries in the two limbs was taken as a measure of the electrophoretic mobility—and hence of the charge on the cells.

In the preliminary experiments some difficulty was experienced in obtaining consistent results. This was traced to slight leakage of air from the partially filled upper spaces past the



stoppers carrying the taps D and E, thus causing mechanical shift of the boundaries due to hydrostatic pressure. The leakages were eliminated by completely filling the spaces with saline and cutting down the length of the column of fluid in B and C to a minimum, thus lowering as much as possible the hydrostatic pressure on the cells. The liquid levels in B and C were balanced before starting the experiment.

If the experiment was continued too long, or if too short agar bridges were used to connect the electrode vessels with the side limbs, acid was found to diffuse from the anode into the cell and cause hæmolysis.

RESULTS.

Hæmolytic experiments.

Colloidal silicic acid in low concentrations (from 0·02 per cent. to 0·12 per cent.) was found to agglutinate suspensions of red blood-corpuscles. On addition of fresh guinea-pig serum the cells underwent lysis; whereas guinea-pig serum heated at 55° C. caused no lysis, the lytic effect being due to complement. Tannic acid in concentrations of 0·004 per cent. to 0·1 per cent. caused similar agglutination, and lysis resulted in the presence of complement. These findings are illustrated by Table I, and are in accordance with the findings of earlier workers on this subject (cited above).

It will be seen that a zone of activity of complement and silicic acid ratios occurs—excess of either of them resulting in a diminution of the hæmolysis.

TABLE I.—*Hæmolysis of Sheep Erythrocytes by Silicic and Tannic Acids.*

	Guinea-pig serum (diluted 1 in 10).								
	Untreated.				Heated (55° C.).				Control—no complement.
	0·05.	0·1.	0·2.	0·3.	0·05.	0·1.	0·2.	0·3.	
Colloidal silicic acid :									
0·02 per cent. . . .	C	p	p	p	0	0	0	0	Agglut.
0·04 ,, . . .	p	p	p	p	0	0	0	0	,,
0·08 ,, . . .	0	p	p	p	0	0	0	0	,,
0·12 ,, . . .	0	0	p	p	0	0	0	0	,,
Tannic acid :									
0·004 per cent. . . .	p	p	p	p	0	0	0	0	Agglut.
0·02 ,, . . .	p	p	p	p	0	0	0	0	,,
0·1 ,, . . .	p	p	C	C	0	0	0	0	,,
Hæmolytic antiserum (horse) 7·5 M.H.D.	p	C	C	C	0	0	0	0	Sl. agglut.
Control (erythrocytes alone) . .	0	0	0	0	0	0	0	0	0

C = complete lysis.
p = partial lysis.

It was shown that the colloidal silicic acid was adsorbed by the red cells during sensitization. The cells and silicic acid were allowed to interact for 1 hour at room temperature, the cells separated by centrifuging and the supernatant fluid and the resuspended cells tested separately. The supernatant fluids were distributed in 0·5 c.c. amounts in tubes, 0·015 c.c. of fresh red-cell sediment was added to each and then complement as usual. The sensitized cells were suspended in saline and complement added. The results are shown in Table II.

TABLE II.—*Adsorption of Silicic Acid by Sheep Erythrocytes.*

		Guinea-pig serum (diluted 1 in 10).				
		0·05.	0·1.	0·2.	0·3.	Control.
Erythrocytes *plus* silicic acid . . .	0·01 c.c. .	p	p	C	C	0
	0·02 ,, .	p	p	p	C	0
	0·03 ,, .	0	0	C	C	0
Supernatant fluid from erythrocytes *plus* silicic acid . . .	0·01 c.c. .	0	0	0	0	0
	0·02 ,, .	p	0	0	0	0
	0·03 ,, .	p	p	C	C	0
Sedimented cells from erythrocytes *plus* silicic acid . . .	0·01 c.c. .	p	p	C	C	0
	0·02 ,, .	p	p	C	C	0
	0·03 ,,	p	p	C	C	0

It will be seen that with the lower concentrations of silicic acid the red cells adsorb all of it, but with the highest concentration a certain amount is left in the supernatant fluid.

In order to determine whether the action of silicic and tannic acids might be due to the hydrogen ion the effects of weak solutions of non-colloidal acids were examined. In certain concentrations such solutions were lytic by themselves, the lower limits in our tests being approximately 0·1 c.c. of $N/100$ hydrochloric or acetic acids per 0·5 c.c. of red-cell suspension and 0·3 per cent. of boric acid. In quantities slightly lower than these limits there appeared to be no hæmolytic effect in the presence of complement.

In order to determine whether acids in the colloidal state were effective a series of organic acids was examined. 1 per cent. alcoholic solutions of stearic, abietic, myristic, pelargonic, azelaic, adipic, tiglic and picric acids

TABLE III.

Experiment.	Time (hrs.).	Current (m.a.).	Control R.B.C. alone.		Immune body.			Cobra venom.			Silicic acid.		
			S.R. (cm./hr.).	Migration (cm.).	Amount (M.H.D./c.c.).	S.R. (cm./hr.).	Migration (cm.).	Amount (per cent.).	S.R. (cm./hr.).	Migration (cm.).	Amount (per cent.).	S.R. (cm./hr.).	Migration (cm.).
1	4·5	3	0·28	1·1									
			0·28	0·9									
			0·31	0·85									
2	4	7·5	0·23	1·25									
			0·25	1·40									
			0·29	1·55									
			0·24	1·35									
3	6	4	0·25	1·0	25	0·28	0·9	..	..	..	0·2	0·32	0·9
4	4·5	3	0·23	0·4	25	0·26	0·5	..	..	..	0·2	0·35	0·0
											Very marked agglutination		
5	5	4·5	0·22	1·2	..	..	..	..	..	..	0·05	0·44	0·55
											Very marked agglutination		
6	5	4·5	0·30	1·25	..	..	..	..	..	..	0·03	0·36	1·05
7	5	5	0·29	0·9	25	0·27	0·7	0·05	0·28	0·9	0·015	0·43	0·9
8	5	4·5	0·31	0·9	..	..	..	..	..	..	0·01	0·40	0·8
9	5	5	0·30	0·95	25	0·27	0·8	0·05	0·29	0·9			
10	5	5·5	0·29	0·85	..	..	..	..	..	..	..	..	..
11	5	4	0·29	0·8	..	..	..	..	..	..	..	..	..
12	5	5	0·29	0·9	..	..	..	..	..	..	..	..	..

S.R. = Sedimentation rate due to

were added to physiological saline to give 0·1 or 0·2 per cent. colloidal suspensions. These were first tested in amounts varying from 0·05 to 0·5 c.c. with 0·5 c.c. of red-cell suspension to determine their lytic effect *per se*.

The addition of complement to erythrocytes treated with these acids in amounts just below the lytic dose was shown to be without lytic effect in every case.

It appeared, then, that neither the hydrogen ion as such nor colloidal nature alone is responsible for the action of silicic and tannic acids in sensitizing sheep red cells to the action of complement. Further evidence that hydrogen ion concentration is not the governing factor is provided by the fact that the dissociation constants for silicic and tannic acids are very different, 3×10^{-10} and $3{\cdot}7 \times 10^{-5}$ respectively, whilst those of the organic acids listed above are reasonably close to that of tannic acid, ranging from 1·1 to $6{\cdot}6 \times 10^{-5}$.

III.

Tannic acid.			Gallic acid.			Sulphur.			Gold.		
Amount (per cent.).	S.R. (cm./hr.).	Migration (cm.).	Amount (per cent.).	S.R. (cm./hr.).	Migration (cm.).	Amount (c.c.).	SR. (cm./hr.).	Migration (cm.).	Amount (c.c.).	S.R. (cm./hr.).	Migration (cm.).
0·025	0·41	1·1									
Marked agglutination											
0·16	0·41	0·65									
Marked agglutination											
0·008	0·31	0·95									
0·008	0·30	0·85									
			0·005	0·29	0·80						
			0·01	0·30	0·85						
..	..	..	0·025	0·28	1·10						
			0·05	0·31	0·80						
						0·25	0·29	0·9			
..	..	..	..	..	..	0·50	0·30	0·75			
									0·25	0·29	1·0
..	..	..	..	..	..	..	..	..	0·50	0·29	0·9

gravity. m.a. = milliamperes.

Other colloidal solutions were similarly investigated, and found to be incapable of sensitizing erythrocytes to the action of complement. These included stannic oxide both in acid and in alkaline solution, starch, colloidal sulphur, and colloidal gold. Gallic acid was also found to have no effect in sensitizing red cells.

Cataphoresis experiments.

When sheep red cells in 10 per cent. suspension were treated with silicic or tannic acids in amounts known to cause lysis in the presence of complement, there was no detectable alteration of the rate of migration of the cells (within the limits of experimental error) except in cases where very marked agglutination occurred. In these cases (usually with the higher concentrations) either the suspension deposited completely or else there was an apparent lowering of the normal negative charge carried by the cells, as shown by a smaller migration than the control. Treatment with hæmolytic antiserum, cobra venom, colloidal sulphur or gold or gallic acid did not alter the rate of migration of the cells.

Typical results are illustrated by the data collected in Table III.

Experiments 1 and 2 illustrate the degree of consistency which can be obtained. The values indicate the migration of the cells towards the anode, the bracketed values being those obtained with the same suspension in different U-tubes connected in series. It can be assumed that the voltage gradients in all the U-tubes are very nearly equal, since the values for the migrations of identical suspensions are in good agreement. Experiment 3 shows the lack of effect of the addition of hæmolytic antiserum and of an amount of silicic acid which did not cause agglutination, the migration in each case being the same as with the control, whilst in Experiment 4 an amount of silicic acid causing marked agglutination is seen to have reduced the migration of the treated cells to zero. From a consideration of the sedimentation-rates due to gravity alone it is seen that the clumps of agglutinated cells settle more rapidly than unagglutinated cells (Table III). Where the agglutination is not too great, however, it is still possible to observe the electrophoretic mobility (Experiments 4 and 5). Similar effects are to be seen in the remaining experiments. In each case the migration was towards the anode, indicating a negative charge on the corpuscles.

DISCUSSION.

The results described here would suggest that there is little evidence for the view, often expressed, that agglutination and cytolysis follow as the result of the alteration of the charge on the surface of the cells involved. Such diverse substances as hæmolytic antiserum, silicic and tannic acids are capable of causing the agglutination of erythrocytes and of sensitizing them to the hæmolytic action of complement, without, apparently, altering the surface charge carried by the cells.

Reiner and Fischer (1929) suggested that the effect of tannic acid in sensitizing erythrocytes was due to a " dehydration ", with conversion of the stable

hydrophilic system to an unstable hydrophobic state readily coagulated by the presence of salts. A similar suggestion was put forward by Sumner and Howell (1936) to explain the action of the hæmagglutinin (concanavalin-A) of the jack bean. Brown and Broom (1935), using as a criterion the amount of adsorption of erythrocytes by filter-paper, considered that an immune serum specifically lowers the charge on the sensitized cells. Abramson (1930), on the other hand, found that although certain concentrations of immune sera caused a lowering of the cataphoretic mobility of erythrocytes, hæmolysis in presence of complement occurred before there was any observable change in the mobility. He stated that sensitization to hæmolysis by sera does not primarily affect the mobility of the cells.

The present findings confirm those of Abramson for hæmolytic antiserum, and extend them to the cases of silicic and tannic acids and of cobra venom in concentrations which sensitize the cells to the action of complement. In the case of silicic and tannic acids higher concentrations (causing very intense agglutination) lower the surface charge on the cells and hence reduce their mobility in the electric field. The mechanism of hæmolysis, however, remains quite obscure, although it seems certain that surface changes due to adsorption at interfaces (see Schulman and Rideal, 1937) must be involved.

The fact observed here that erythrocytes may be sensitized to hæmolysis by complement without any observable change in the electric charge, whilst agglutination is often accompanied by a reduction of the charge, is in keeping with the view of Landsteiner and van der Scheer (1929) and of Mudd and Mudd (1926) that the phenomena of hæmolysis and hæmagglutination are independent, and involve the interaction of different chemical constituents of the red cells, with different specific antibodies or the same non-specific agent.

SUMMARY.

1. The hæmolytic effect of colloidal silicic and tannic acids in conjunction with serum-complement has been confirmed.

2. Various other acids, both electrolytes and colloids, were found to be without such action ; colloidal starch, sulphur and gold were also inactive.

3. Concentrations of silicic acid, tannic acid, hæmolytic antiserum and cobra venom sufficient to cause sensitization of erythrocytes were found to have no observable effect on the electrokinetic mobility of the cells.

4. Higher concentrations of silicic and tannic acids, sufficient to cause strong agglutination of red blood-corpuscles, were found to lower the surface charge of the cells.

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THE PROPERTIES OF ANTIGENIC PREPARATIONS FROM *BRUCELLA MELITENSIS*: III. THE BIOLOGICAL PROPERTIES OF THE ANTIGEN AND THE PRODUCTS OF GENTLE HYDROLYSIS.

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IN the first two papers of this series (Miles and Pirie, 1939*a*, *b*) we described the preparation and properties of the antigenic complex of *Br. melitensis* in two states of aggregation. The smaller of these complexes was called AP and the larger, which contained in addition a mixture of phospholipoids and a protein-like material, was called [PLAPS].

In this paper, preliminary conclusions about the constitution of AP based on a study of its hydrolysis are drawn, and some biological properties of the antigenic complexes and their breakdown products are described.

HYDROLYSIS OF AP BY DILUTE ACID.

The substance which remains after the removal of the labile phospholipoid (PL1) and of the substance S, and after disaggregation, is still relatively complex. Besides the formyl-amino compound, which will be described in a later

paper, it contains bound phosphate and a material which may at present be referred to as another phospholipoid (PL2).

The hydrolysis of AP has been followed in a number of different ways, namely, by noting—(1) loss of serological activity, of toxicity and of the power to produce lesions in hypersensitive animals ; (2) loss of characteristic physical properties ; (3) liberation of PL2 ; (4) liberation of free phosphoric acid ; (5) the appearance of amino-groups and of free formic acid ; and finally by the isolation of low molecular weight products after complete hydrolysis. These criteria of hydrolysis have been arranged roughly in the order of the severity of treatment with acid to which they are appropriate.

Hydrolysis for five minutes at 100° with $N/10$ hydrochloric acid will reduce the serum precipitation end-point of a 0·5 per cent. solution of AP to 1 per cent. of its starting value. In this time only about a third of the hydrolysable phosphorus has been set free and there is no precipitation of PL2, though, as will be shown later, the PL2 has been liberated. If the hydrolysis under these conditions is continued for about 45 minutes, the solution becomes turbid and a flocculent white precipitate of PL2 separates. At the same time the liberation of inorganic phosphate is complete. The PL2 fraction represents about 20 per cent. of the starting weight of AP, but it contains about one-half of the initial 0·6 per cent. of phosphorus ; the other half is now present as free phosphoric acid.

These steps in the hydrolysis are illustrated in Table I. Free phosphate was estimated by the method of Kuttner and Lichtenstein (1932) ; if the material is not incinerated it is necessary to separate the phosphate from AP and its breakdown products, for they give a precipitate with the molybdate reagent. The following procedure for estimating phosphorus distribution is the best that has been found, although, as can be seen from Table I, recovery is incomplete. After various periods of boiling, 5-mg. samples were withdrawn from a solution of AP in $N/14$ HCl. The appearance of the fluid is described in Column 2. (Table I). The sample was diluted to 1 c.c., precipitated with 10 c.c. of alcoholic 0·5 per cent. calcium chloride and centrifuged after 2 hours. A drop of concentrated ammonia precipitates free phosphate from the supernatant, and this calcium phosphate, together with some calcium carbonate, was centrifuged off after two hours. The phosphorus content, measured without incineration, is expressed as a percentage of the original AP in Column 3. The first alcohol precipitate was suspended in 2 c.c. of water, centrifuged after an hour, and the phosphorus content of both solid and fluid was measured after incineration. Column 4 gives the phosphorus content of the fluid and Column 5 that of the

TABLE I.—*The Effects of Acid Hydrolysis on AP.*

(1) Time of hydrolysis (minutes).	(2) Appearance of the fluid.	(3) Percentage of initial weight of AP found as inorganic phosphorus.	(4) Percentage of initial weight of AP found as "AP" phosphorus.	(5) Percentage of initial weight of AP found as PL2 phosphorus.	(6) Serum precipitation end-point.
0	Faintly opalescent	0	0·50	0	1 : 2,000,000
5	Clear	0·03	0·35	0·10	1 : 440,000
10	Faint turbidity	0·06	0·20	0·20	1 : 10,000
45	Precipitate	0·14	0·05	0·21	1 : 200

solid ; the latter represents phosphorus in the form of free PL2, and the former represents unhydrolysed and very slightly hydrolysed AP.

A sample of fluid was prepared as if for the analysis in Column 4 (Table I), *i. e.* containing AP and the phosphorus-free alcohol-precipitable hydrolysis products of AP. This was tested serologically and Column 6 shows the end-points found. It is clear that the loss of precipitability parallels the liberation of PL2 more closely than it parallels the liberation of the inorganic phosphate. The power to inhibit the reaction between equivalent mixtures of AP and antibody disappears less rapidly. The test was made by incubating dilutions of hydrolysed fractions for 1 hour at 56° C. with antiserum equivalent to 1 : 360,000 AP (*i. e.* about 6 × the end-point concentration), and then testing for free antibody by adding 1 : 360,000 AP. Fractions hydrolysed for 10 minutes or less have approximately equal precipitin and total-inhibition end-points. After ten minutes' hydrolysis the inhibition titre is the greater, and eventually substances are left in the soluble part of the hydrolysate which inhibit at 1 : 16,000, but are not precipitated at 1 : 200. The nature of this simple inhibiting hapten will be discussed in a later paper.

After hydrolysis for periods up to an hour in the manner described above, a product is obtained which is free from phosphorus and serologically inert, but which is still large enough to be retained during dialysis in Cellophane. It will pass through collodion membranes which, though more open than Cellophane, will retain the original AP. In the presence of salts it can be precipitated by alcohol, and it can still be precipitated by phosphotungstic and phosphomolybdic acids, but no longer by ammonium sulphate. The fact that AP inactivated by acid or alkali can no longer be precipitated with ammonium sulphate is of great value in the purification of old preparations, for any material which has been inactivated by rough handling will presumably be removed by this purification process.

AP from which PL2 and phosphate have been removed still contains the material that can be estimated as sugar by the orcin method and, after treatment with alkali, will give a colour with *p*-dimethylaminobenzaldehyde similar to that given by the amino-sugars (Elson and Morgan, 1933). Like AP, the molecule can be acetylated with acetic anhydride and pyridine ; two acetyl groups are introduced for each formyl group originally present. Judging from the ease with which they can be removed with alkali, these are O-acetyl groups. These and other chemical properties of AP will be described more fully in a later paper.

The ease with which AP is hydrolysed by acids can be used as an argument in favour of its chemical homogeneity. The only other substance that is likely to be present, and which would yield phosphate on hydrolysis, is nucleic acid ; both yeast nucleic acid and the nucleic acid derived from *Br. melitensis* are approximately five times as resistant to acid hydrolysis as is AP. It is very unlikely that the labile phosphate is due to contamination with a nucleic acid and, furthermore, it has proved impossible to demonstrate the presence of pentose or desoxy-pentose in purified preparations of AP.

Agar is well known to be relatively unstable towards acids, but the instability of AP is too great for this to be of value as a means of removing agar from preparations in which both are present.

The Insoluble Material Liberated by Acid Hydrolysis.

The liberation of the insoluble white material called PL2, on gentle hydrolysis of the PL1-free antigen is in many ways the most interesting feature of this study. A substance which is presumably identical with our PL2 was described by Huston *et al.* (1934), and by Damboviceanu *et al.* (1938) but the insoluble material which Morgan (1937) observed as a hydrolysis product of *Bact. shigæ* is probably not closely related.

The degree of hydrolysis, sufficient to liberate PL2 from the rest of the molecule, is insufficient to make it flocculate but, as has already been stated, if the acid mixture is precipitated with alcohol, the PL2 precipitates also and does not redissolve in water. When prepared by the mildest possible hydrolysis, *i. e.* 10 minutes in $N/14$ acid at 100° C., it is a gelatinous solid insoluble in water, but readily soluble in dilute alkali; in this respect it resembles Morgan's product but, unlike that product, it gives no protein reactions.

PL2 prepared by hydrolysis sufficiently vigorous to cause flocculation (*i. e.* 1 hour in $N/10$ acid at 100° C.) constitutes 20–26 per cent. of the PL1-free and disaggregated antigen. It separates as opaque amorphous particles, many of which stick firmly to the walls of the vessel in which the hydrolysis is carried out. It is insoluble in dilute acids and alkalis, and in every organic solvent that has been tried except phenol and meta-cresol, and in these solvents solution is incomplete. In spite of this insolubility, the material may be referred to as a lipoid, for it has a somewhat waxy feel, and it can be prepared as a birefringent film when orientated by squeezing a particle between two glass plates. More definite justification for classifying PL2 as a phospholipoid is obtained from its high carbon content and from its behaviour on oxidation with nitric acid. The products of oxidation will be described in a later paper. PL2 gives on combustion an amount of residue greater than would be expected from its phosphorus content on the assumption that this is weighed as HPO_3. On account of its insolubility, the percentage of residue is probably not very significant, for insoluble impurities may easily have been gained during the manipulations. The analytical figures on our preparations have fallen within the following ranges: C 60–62 per cent., H 9·4–10·0 per cent., N 4·3–4·6 per cent., P 1·4–1·6 per cent., and residue 5–8 per cent. These figures are similar to those given by Vanghelovici *et al.* (1938), except that these workers doubt the presence of phosphorus, which we find to be invariably present.

PL2, isolated by gentle acid hydrolysis and in the state in which it can be dissolved in dilute alkali, contains about one-third of its weight of non-lipoid material, which can be removed by more vigorous hydrolysis. This material resembles the water-soluble material that can be precipitated by alcohol from AP solutions that have been hydrolyzed sufficiently vigorously to yield PL2 in the insoluble state. There is no reason to think that this material is not removed gradually from the PL2 during acid hydrolysis, but the amount of AP available has been insufficient for a proper characterization of the fractions that are successively split off.

The ultra-violet absorption spectrum of the soluble complex of PL2 has been determined and it is set out in Fig. 1, together with the absorption spectra of AP and AP from which PL2 has been removed. It is obvious that PL2 is responsible for the greater part of what little absorption AP solutions show, and that

the formylated amino-polyhydroxy component of AP (Curve 3, Fig. 1) contributes but little to the whole absorption. In Fig. 1 the spectrum of the substance S that is present in [PLAPS] is also recorded, and the strong absorption at 275 mμ. characteristic of aromatic nuclei is consistent with our view (Miles and Pirie, 1939*a*) that S is, in part at least, protein. Rough colorimetric estimations of tyrosine and tryptophan in S show that together they represent not more than 5 per cent. of the S molecule. Other proteins which have a similar tyrosine and tryptophan content absorb U.V. light at 275 mμ. to the same extent as S or to a slightly smaller extent (*cf.* Sjögren and Svedberg, 1930). Pennell and Huddleson (1937) concluded from a study of humin formation that the *Br. melitensis* antigen contained 19 per cent. of tryptophan and 8·5 per cent. of tyrosine. The feeble U.V. absorption of AP is a very strong argument against this conclusion ; furthermore AP gives none of the

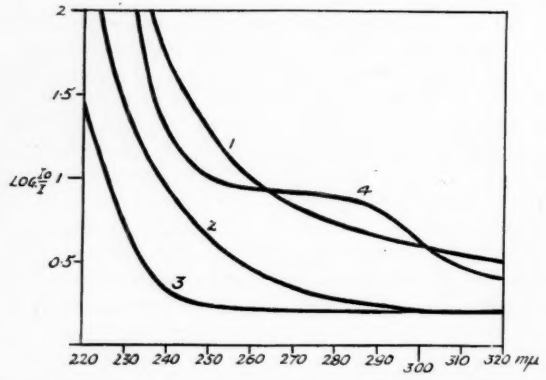

Fig. 1.—Absorption spectra on solutions all measured in a cell 2 cm. deep. Curve 1 : 0·02 per cent. solution of PL2 in *N*/150 sodium hydroxide. Curve 2 : 0·04 per cent. solution of AP in water. Curve 3 : 0·10 per cent. solution of AP from which the PL2 has been removed by gentle acid hydrolysis. Curve 4 : 0·02 per cent. solution of the substance S.

colour reactions for either amino-acid. Since the native antigen contains at most 10 per cent. of S, it cannot contain more than 0·5 per cent. of tryptophan and tyrosine.

BIOLOGICAL TESTS.

There is no laboratory animal sufficiently susceptible to fatal brucellosis to permit an accurate assay of the immunizing properties of bacterial fractions, on the basis of a quantal response (*i. e.* death or survival of the animal). The dose of living *Brucella* that kills the mouse within 2–3 days was found to be of the same order of size as the lethal dose of dead bacilli. Such doses kill more by intoxication than by invasion, and it is immunity to intoxication rather than immunity to infection which would be thereby tested. In the search for an immune reaction of living tissue, an attempt was made to assay the neutralizing effect of fractions on the bactericidal power of the defibrinated blood of immune rabbits. A sealed tube technique (Miles and Misra, 1938)

was used. The antibodies had a strong opsonic effect, but no accurately measurable bactericidal power was detected in the blood-bacterium mixtures, and the matter was not investigated further. It is possible that the substitution of open aërated tubes might be more successful, for human blood was found to have a definite killing effect on Y6 after 24 hours' incubation in unagitated open tubes (von Haebler and Miles, 1938).

The *in vivo* antigenic powers of fractions were measured by the production of agglutinins, skin reactivity in sensitized guinea-pigs and rabbits, and immunizing powers against toxic doses of AP.

Antigenic Powers.

AP.

One tenth of a mg. injected intradermally sensitizes rabbits' and guinea-pigs' skin to AP in 7 days, and produces bacterial agglutinin titres up to 1 : 320 in 10 to 21 days. Intramuscular injections are as effective as intradermal (2 batches, each of 6 guinea-pigs, compared). Precipitins are not formed after single injections. The agglutinating sera give marked prozones. Intravenous injection is less effective; sera with average titres of from 1 : 4 to 1 : 64 are produced in 5 to 10 days after injecting 1 mg. intravenously (8 rabbits), and 1 : 256 after a 5-mg. dose (2 rabbits).

[PLAPS.]

Single intradermal doses of 0·1 mg. produce agglutinin titres of 1 : 3000 and feeble precipitating sera for AP. Intravenous doses of 1 mg. give agglutinin titres of 1 : 1000.

TABLE II.—*Antigenic Powers of [PLAPS], PLAPS and AP by Different Injection Routes. Rabbits from 2·0–2·4 kg. Mean* Br. melitensis *Reciprocal Agglutinin Titres Recorded.*

(1).	(2).	(3).	(4).	(5).	(6).	(7).	(8).
Injection route:	Intravenous.		Intradermal.			Intradermal.	
Antigen:	[PLAPS.]	AP.	[PLAPS.]	AP.	AP.	PLAPS.	AP.
Dose (mg.):	1	1	0·2	0·4	10 × 0·04	0·2	0·2
No. of rabbits:	4	4	3	3	3	4	3
Time (days):							
3	..	..	12	2	<2	..	..
4	320	5	..	..	..	140	53
7	..	..	3000	190	310	260	184
10	..	..	..	46	270	194	293
16	1000	16	680	56	600	170	280
24	..	..	..	..	..	72	122
36	180	20	..	..	..	..	..

The comparatively feeble antigenicity of AP (see Cols. 2 and 3, Table II) introduced by the intravenous route suggests a rapid elimination compared with that of the larger and less diffusible [PLAPS] aggregates. Columns 4 and 5 show the antigenic superiority of 0·2 mg. of [PLAPS] over 0·4 mg. of AP,

injected intradermally in 0·2 c.c. volume. But, by splitting the 0·4-mg. dose of AP into ten 0·04-mg. doses each in 0·2 c.c. of saline and injecting these into ten different parts of the skin on the same day, the antibody response (Col. 6) is greatly enhanced. The presence of PL1 and S does not materially enhance the antigenicity of AP as measured by these tests. We have already described (Miles and Pirie, 1939*b*) the disaggregation of [PLAPS] to a substance called PLAPS, which still contains PL1 and S, by incubation with dodecyl sulphate. In Columns 7 and 8 PLAPS is compared with AP. The maximum response to PLAPS occurs earlier than that to AP, but the two maxima are of the same size, *i. e.* in this respect AP behaves like PLAPS. It is possible that AP owes its antigenicity to contamination with PLAPS, since the presence in it of much less than 10 per cent. of the original amount of PL1 and S would not be detected by the chemical tests that have been used. The antibody response to [PLAPS] is much greater than that to PLAPS—a result that must be attributed to a difference in the state of aggregation, for the two substances are chemically similar.

Skin Tests.

Skin hypersensitivity was not easily induced by the injection of small or massive doses of dead bacilli, and was only feebly induced by passive immunization with strong antisera. A dose of 200×10^6 living organisms induces within a month a good reactivity in rabbits that is definitely associated with the precipitating power of the serum for AP. Animals with an agglutinin titre of over 1 : 640 react distinctively with AP, but unless the serum, diluted 1 : 5, gives visible precipitates with AP, the skin reaction is likely to be slow and small and, with more complex fractions, indistinguishable from the control reaction. In two rabbits the accidental induction of a prolonged negative phase by a second dose of living organisms two months after the first resulted in a complete loss of skin sensitivity. Poor reactions occurred in four guinea-pigs that subsequently died of brucellosis, and it is probable that hypersensitivity is low in progressive disease.

The area of an AP lesion (calculated from $\pi dD/4$, where d and D are its minor and major axes) is a fair indication of its state, but with more complex fractions the height of the lesion also was a distinctive factor. Height (h) could be estimated to within 0·5 mm. with sufficient accuracy, and since the lesions were mainly flat-topped with steep edges, the expression $h(\pi dD/4)$ is a reasonable estimate of the volume. Fig. 2 records areas, figs. 3–5 volumes, plotted against time. The fractions were injected intradermally in 0·2 c.c. volumes. The titration of fractions in sensitized rabbits gave rise to no distinctive end-points like those recorded by Pennell and Huddleson (1937), and it is doubtful whether, in view of the high variability we found in the response of immunized rabbits, it is possible to make any absolute measurement of reactivity, or any reliable comparisons of reactivity in animals sensitized with *Br. abortus* and *Br. melitensis* respectively. But the relations of the day-to-day changes in sensitized or normal animals were similar in all those tested, and they depended on the nature of the fraction injected. All series of fractions were tested on groups of 4, 6 or 8 animals.

AP.

The 1-day lesion in a *normal* rabbit from 0·1 mg. AP is pink and very slightly indurated over an area of 250 mm.^{2}, and has disappeared completely by the 4th day. Guinea-pig reactions are similar, 200 mm.^{2} in area, and disappear in 6 days. In *sensitive* animals, 0·1 mg. of AP gives in 1 day reactions up to 700 mm.^{2}, increasing for 2 more days, then decreases gradually until

the lesion has gone, usually in 12–25 days. But the induration is maximum in 4 days, when the swelling may be up to 5 mm. in height. Commonly the centre of the lesion is hæmorrhagic or pus-yellow, and in 4 days a scab of 75 mm.² forms, under which the swelling subsides, leaving a deep sterile scabbed ulcer with raised wide ring of indurated and inflamed skin. The lesion produced by 0·01 mg. is about 100 mm.²; it seldom ulcerates, though occasionally sterile abscesses form and later dry up. The ulcerated lesions persist for several weeks. Fig. 2 shows the lesion-areas averaged for 8 rabbits sensitized with Y6. AP lesions are maximum in 1 or 2 days, but the height decreases more slowly than the area; the area attained is proportional to the precipitin titre of the AP fraction tested. The [PLAPS] and PLAPS reactions are delayed and resemble that of whole bacteria (killed Y6 suspensions).

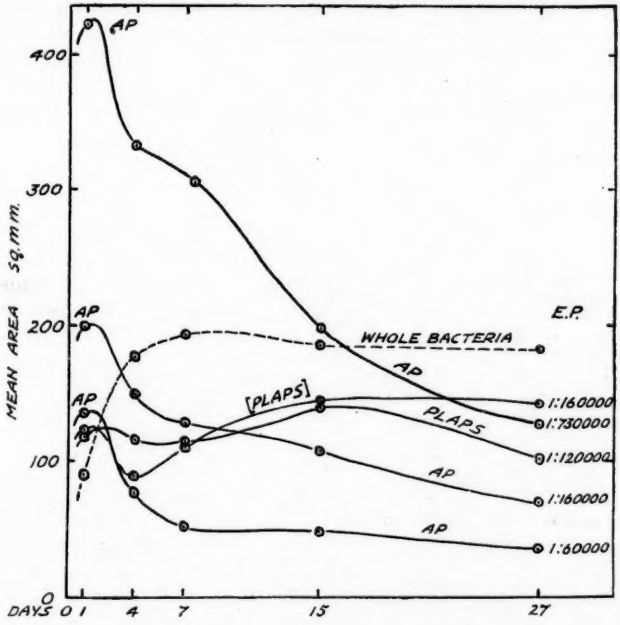

FIG. 2.—Average skin-lesion areas in 8 sensitized rabbits.

In Fig. 3 the average volumes of the lesions caused by 0·2 mg. [PLAPS] and AP in 4 sensitized and 4 normal rabbits are compared with those caused by 2×10^8 dead Y6. The AP lesion rises to a single peak on the 3rd day and decreases steadily till the 18th. [PLAPS] produces three peaks. The first is maximum in 1 day and is inconstant in its appearance. The second and third peaks have appeared in 11 of 12 rabbits tested; they consist of a sharp maximum on the 3rd or 4th day, and a prolonged response that is maximum on the 8th or 10th day. In normal rabbits [PLAPS] injections produce, in 4–7 days, the beginning of a consistent and definite prolonged response, maximum about the 10th day; the Y6 curve for whole bacilli shows a similar slow rise with a characteristic prolonged response.

The curves for [PLAPS]—native, disaggregated with dodecyl sulphate, and treated with acetic acid to produce AP (all derived from one specimen)—are exemplified in Fig. 4, typical of 4 rabbits so tested. The native [PLAPS] shows the initial rise, a sharp second peak at 4–5 days and the slow rise, commencing on the 10th day. Disaggregated [PLAPS] produces a more rapid reaction, in which the initial rise is apparently swamped in a single large peak, and in which the rise and fall of the third peak is relatively rapid. The AP produces a single peak with an early 2-day maximum, with a lag in its general decline on the 5th day. With the degradation from whole bacterium to AP there is a gradual disappearance of the prolonged response both in sensitized

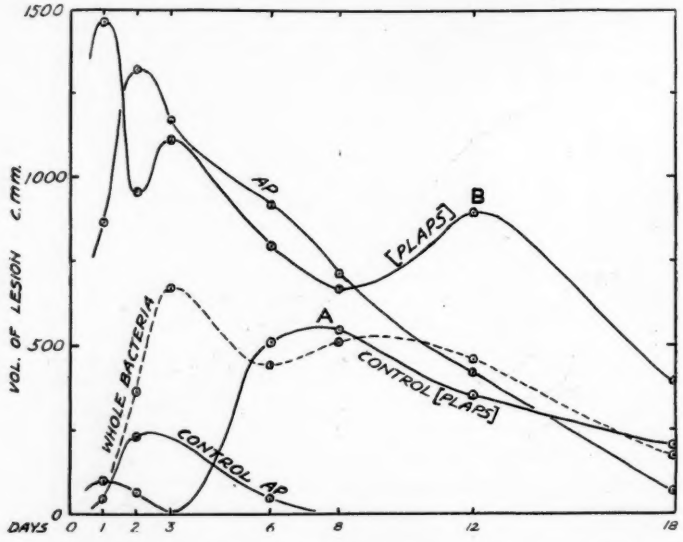

FIG. 3.—Average skin-lesion volumes in 4 sensitized and 4 control rabbits, 0·2 mg. doses.

and control animals, and an increase in the intensity and the rapidity of the early response in the former. Y6- and [PLAPS]-lesions of 0·2 mg. seldom ulcerate, but hæmorrhagic centres with subsequent scab and ulcer formation are common in AP-lesions.

The immediate response of AP and the 3–4-day response of [PLAPS] is referable to the amount of reacting substance readily available; presumably [PLAPS] slowly liberates AP into the tissues of the skin. The prolonged response that follows this is less readily explained. It occurs in the controls, and may equal the response of the sensitized animal in volume. In 5 control rabbits its appearance coincided with the appearance of measurable agglutinins in the serum and the development of skin sensitivity to AP. It is reasonable, therefore, to suppose that this antibody, formed in response to antigen at first liberated from the skin-bleb, sensitizes the skin, which responds several days

later to antigen still being slowly liberated from the site of injection ; and that the prolongation of this secondary response is due to the continuous slow breakdown of the [PLAPS] in the skin tissues. In the sensitized animal its occurrence cannot be due to the first appearance of circulating antibodies, or to any significant change in antibody level, for neither precipitin nor agglutinin titres were altered significantly in the first 10 days after the intradermal injections. Both chemical and serological tests have shown that aggregated [PLAPS] is a relatively unstable substance, readily disaggregating, and even liberating a small amount of AP. The occasional initial peak might then be due to AP, the second to disaggregated [PLAPS] and the slow response to the gradual breakdown of [PLAPS] aggregates. In the controls, AP has no effect,

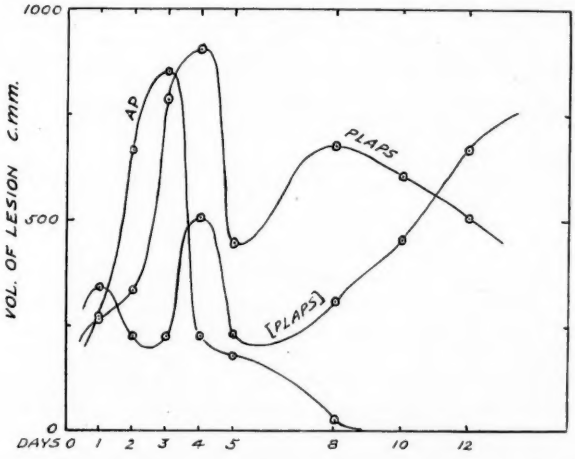

FIG. 4.—Skin-lesion volumes with native antigen, disaggregated antigen and AP. Typical of 4 sensitized rabbits.

and disaggregated [PLAPS] gives only a small prolonged response, so that its presence in [PLAPS] serves only to enhance the characteristic [PLAPS] effect of a large prolonged response.

The 9-day response of disaggregated [PLAPS] in Fig. 4 is not referable to the size of fraction disaggregated, for PLAPS particles are smaller than particles of AP as prepared by our standard technique. The readiness with which dodecyl sulphate-treated AP reaggregates in the presence of agents like acetic acid or ammonium sulphate may have its parallel in PLAPS, which, may to a certain extent reaggregate in the presence of body-fluids. But it is possible that the prolonged response may in part be due to the PL1 fraction.

The further degradation of AP by acid hydrolysis at 100° C. with $N/14$ HCl produces no change in the shape of the AP time-response, but a marked reduction in intensity that is paralleled by a drop in toxicity (see Table IV) and precipitin end-point (Fig. 5).

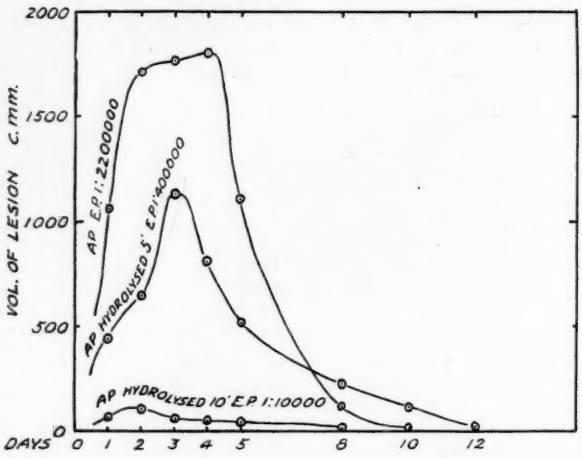

FIG. 5.—Skin-lesion volumes with hydrolysed AP, typical of 5 sensitized rabbits. AP, hydrolysed 17 minutes, of EP<1 : 200, produced no reaction. Two control rabbits gave negative results with all fractions.

The Immunizing Power of AP.

Single small doses of AP confer some protection against subsequent lethal doses of AP. Table III summarizes the result of one experiment. Three batches of 20 mice (Batches 2, 3 and 4) were injected intraperitoneally with 0·001, 0·01 and 0·1 mg. AP respectively, and a fourth batch (Batch 1) kept as control. After 17 days each batch was injected with AP, 2 mg. and 0·4 mg., each into 10 mice. The doses were calculated to produce in the control mice death-rates symmetrically placed above and below the 50 per cent. killing level. The protective power of the immunizing doses may be gauged from the calculated weight of AP required to kill 50 per cent. of the mice in each batch. This weight is the LD50. Compared with the LD50 of control

TABLE III.—*The Immunizing Power of AP against Toxic Doses of AP.* (*Gaddum's Notation* (1933).)

Test batch (2 × 10 mice)	(1.)	(2.)	(3.)	(4.)
Immunizing dose (mg.)	0·000	0·001	0·010	0·100
Test dose (mg. 10 mice)	2·0	2·0	2·0	2·0
p'	1·0	1·0	0·6	0·2
Test dose (mg. 10 mice)	0·4	0·4	0·4	0·4
p'	0·2	0·0	0·1	0·0
b	4·37	6·94	2·20	2·01
$m(b = 3·4)$	$\bar{1}$·781	$\bar{1}$·952	0·140	0·494
LD 50 (mg.)	0·60	0·90	1·38	3·12

Tests.	Ratio toxicities.	$M \pm \lambda_M$.	M/λ_M.
(1) and (2)	1·48	0·171 ± 0·193	0·89
(1) and (3)	2·29	0·359 ± 0·149	2·41
(1) and (4)	5·16	0·713 ± 0·174	4·10

Batch 1 the increase in LD50 in Batch 2 is not significant ; but with increasing immunizing dose the protection is increased until, with an initial dose of 0·1 mg., a significant degree of immunity is conferred.

Toxicity of Fractions.

The toxicity in mice of the following fractions was compared (see Table IV) : (1) and (2), acetone-dried bacteria and a tryptic digest (*cf.* Raistrick and Topley, 1934) of these bacteria ; (3) and (4), [PLAPS] and disaggregated [PLAPS] ; (5) and (6), [PLAPS] and AP ; and (7) and (8), AP and AP after hydrolysis with $N/17$ HCl at 100° C. for 10 and 17 minutes ; only the first two of the last set are recorded, since AP hydrolysed for 17 minutes was non-toxic in 3 mg. doses.

TABLE IV.—*Comparisons of Toxicity of Various Fractions of* Br. melitensis. *Each dose tested in* 10 *mice.* (*Gaddum's Notation* (1933).)

	Pairs of fractions tested.							
	(1) Whole bacteria. (2) Tryptic digest.		(3) [PLAPS.] (4) PLAPS.		(5) [PLAPS.] (6) AP.		(7) AP. (8) Hydrolysed AP.	
Range of mouse weights (g.)	25–30		24–30		22–27		24–30	
	(1.)	(2.)	(3.)	(4.)	(5.)	(6.)	(7.)	(8.)
Dose (mg.)	15	15	3	3	5	5	3	3
p'	0·8	0·9	0·4	0·7	0·5	1·0	0·7	0·1
Dose (mg.)	5	5	0·6	0·6	1	1	0·6	0·6
p'	0·2	0·1	0·0	0·1	0·3	0·7	0·2	0·0
b	2·43	3·51	2·88	1·53	0·75	2·71	1·95	1·63
Mean b	2·94		1·98		1·36		1·85	
m	1·05	1·05	0·677	0·287	0·533	$\bar{1}$·454	0·211	1·144
LD50 (mg.)	11·2	11·2	4·8	1·9	3·57	0·35	0·16	1·40
$M \pm \lambda_M$	0·0 ± 0·169		0·389 ± 0·25		1·10 ± 0·502		$\bar{1}$·07 ± 0·42	
Ratio toxicities	1·00		2·45		12·6		0·118	

Two doses of each fraction, in 0·25 c.c. volumes, were injected intraperitoneally, each into 10 mice. Deaths were observed up to 7 days, and intercurrent bacterial infection was excluded post-mortem. The ratios of the toxicities are calculated as in Gaddum's Example I (Sect. X, Gaddum, 1933). The values of p' obtained in Tests (3), (6) and (8) are not favourable for a reliable estimate of the regression line ; the calculation of the line in (3) and (8) was made possible by taking $y = \pm 2{\cdot}43$ for $p' = 1{\cdot}0$ or $0{\cdot}0$ (fig. 13, Gaddum, 1933). In Tests (7) and (8) the two values of b are by chance close together, but in the three other pairs they were widely divergent ; in all four comparisons, m_1 and m_2, and M were calculated from the mean b of each pair. It is obvious that in the absence of fuller knowledge of the dose-response curves of *Brucella* toxins in mice, and of reliable evidence of the constancy of toxin-sensitivity in the batches of mixed strains of the dealer's mice used, the values of the average 50 per cent. killing doses (LD50) are unreliable.

From Tests (6) and (7) (Table IV) and Test (1) (Table III) it appears that the LD50 of AP for 25–30 g. mice varies from 0·16 to 0·6 mg. The specimens of AP employed were different in each case, the least toxic being made from a large pool of residues. The figures may reflect merely the lack of homogeneity of the test mice, but it is possible that our present criteria of AP purity (no detectable PL1 or S, a typical optimal spread in the precipitin test, with the limiting end-point of 1 in 2·5 million) are insufficiently exact to define

AP, and that chemically or serologically indetectable differences may influence toxicity and antigenicity considerably. There is, for example, an obvious difference in the antigenic powers of the two specimens of AP recorded in Columns 5 and 8 of Table II. In all four pairs of tests recorded in Table IV the variability of preparations was minimized by deriving the pairs of substances each from one typical preparation, so that comparisons of the toxicities of such pairs are valid. The true value of any of the ratios is assumed to lie between antilog. $M - 1{\cdot}96\,{}^{\lambda}M$ and antilog. $M + 1{\cdot}96\,{}^{\lambda}M$—a range that contains 95 per cent. of the likely values of the ratio (Irwin, 1937). Whole acetone-dried bacteria and tryptic digest of the same quantity of bacteria are equally toxic. The whole bacteria were soaked in water at pH 8·5 at 37° C. for one week—a treatment similar to that of the digest except that trypsin was omitted. The results suggest that the toxic principle of *Br. melitensis* is resistant to trypsin. But Tests (5) and (6) show that increases in dispersion are accompanied by increases in toxicity, and that the one may result in the other. A similar difference in dispersion may affect Tests (1) and (2), and the maintenance of toxicity during tryptic digestion may be the result of a cancellation of increasing toxicity due to dispersion, by a decrease due to the digestion of a toxic protein constituent. A series of similar tests of several strains of *Br. melitensis* is required to settle this point.

AP is 12·6 times as toxic as the [PLAPS] from which it is derived. The error of this determination is large, but the true value is unlikely to lie outside the range 1·3–121·3, so that the ratio 12·6 indicates a significant increase in toxicity. The change [PLAPS] to PLAPS produces no significant increase in toxicity. In both changes the dispersion of the substance, and presumably the readiness with which it spreads through and poisons the mouse tissue, is increased. But the increase in toxicity exhibited in Tests (5) and (6) (Table IV) cannot be entirely due to an increase in dispersion, for, if we assume that the specimens of [PLAPS] in (3) and (5) are directly comparable (having LD50's of 4·8 and 3·6 mg. respectively), then AP is more toxic than PLAPS. We have already shown that PLAPS is more highly dispersed than AP, and must conclude that the presence of PL1 and S may in some way mask the toxicity of AP in the PLAPS and [PLAPS] complexes.

Treatment with sodium dodecyl sulphate does not itself lessen toxicity materially, at any rate as far as AP is concerned. The toxicity of AP, AP disaggregated with dodecyl sulphate and AP reaggregated by $(NH_4)_2SO_4$ precipitation was tested as in Table IV. The doses in each case were 3 and 0·6 mg. and the three pairs of values of p' were 0·9 and 0·4, 0·8 and 0·6, and 1·0 and 0·5, yielding LD50's of 0·74, 0·65 and 0·45 mg. respectively. These results show also that the disaggregation of AP has little effect on its toxicity. The LD50 of AP decreased 8·5-fold after 10 minutes' hydrolysis, a decrease in toxicity which lies just on the border of significance as defined above, since the true value is unlikely to lie outside the range 1·3 to 56·2. The loss of toxicity on hydrolysis is marked in the 17-minute hydrolysate, which was non-toxic in 3-mg. doses.

Most mice die within one or two days after injection of *Brucella* toxins. Whole bacteria produce no special post-mortem features; there is a little peritoneal exudate, the liver is pale and

mottled, the spleen congested. In addition, the tryptic digest and [PLAPS] produce in 80 per cent. of the mice large sharply-defined, angular, hæmorrhagic areas on the lung surface. AP produces the same changes, but not so commonly ; the commonest lung change is a fine mottling of pin-point hæmorrhages, and occasionally a diffuse hæmorrhage throughout the tissue. The other characteristic change is a fine hæmorrhagic mottling of the peritoneal surface of the small intestine, most marked in the duodenum and jejunum. Microscopically the lung changes suggest no peculiar toxic agent ; they resemble the hæmorrhagic lesions produced by many rapidly-acting toxic substances.

In rabbits, AP is apparently more toxic than [PLAPS] ; 3 of 8 rabbits died within 18 hours after 1 mg. of AP intravenously, whereas 4 rabbits of similar weight were unaffected by the intravenous injection of 1 mg., and 2 rabbits by the injection of 5 mg., of [PLAPS].

DISCUSSION.

The composition of the native antigen of *Brucella melitensis* has been explored by following the process of its disintegration ; in summarizing this work it will be convenient to begin at the other end. Our description of the process of disintegration has been carried so far as to leave a water-soluble substance that does not diffuse through Cellophane ; it is essentially the polymerized formyl derivative of an amino-compound with about two acetylatable hydroxyl groups for each formyl group. This material is serologically inert, but it is derived from a simple hapten by the loss of a molecule of phosphate for each unit of molecular weight 1400. In sensitized rabbits concentrated solutions of the hapten produce hæmorrhagic reactions, but are not antigenic. The products of hydrolysis of this substance will be described elsewhere.

The next stage in complexity is a stable, well-characterized substance which we have referred to by the symbol AP ; we consider AP analogous to the SSS that have been isolated from *Bact. aertrycke* and *Bact. typhosum* (Raistrick and Topley, 1934 ; Topley *et al.*, 1937), and from *Bact. shigæ* (Morgan, 1937), and to the " complete " antigens isolated by Boivin and his colleagues from a number of Gram-negative bacteria (Boivin and Mesrobeanu, 1935). It is unaffected by boiling or drying, and does not deteriorate on keeping ; it is toxic, antigenic, and precipitates with antisera in a final dilution of 1 : 5,000,000. The simple hapten is derived from AP by the loss of a phosphorus- and nitrogen- containing substance which is referred to by the symbol PL2. We have not been able to produce from AP a toxic, non-antigenic, specifically precipitable hapten analogous to those described by Morgan, for the loss of PL2 is accompanied by a simultaneous but not strictly parallel loss of toxicity, antigenicity and precipitability. AP is an acid with an equivalent weight of about 8000. Its molecular weight can be estimated from the sedimentation constant, and varies between 100,000 and 1,000,000 according to the previous treatment. Aggregation and disaggregation in this range is reversible.

AP is derived from a more highly polymerized material which is combined with a protein-like material that is referred to by the symbol S. When in this stage of complexity it is easily sedimentable on a high-speed centrifuge and is no longer unaffected by drying. Lastly, the native antigen, prepared by the gentlest possible treatment of the bacteria, is this substance combined with one-third to one-quarter of its weight of a mixture of lipoids and phospholipoids (PL1) ; in this state the antigen is unstable and cannot be dried,

frozen or treated with organic solvents without modification. These treatments do not result in a complete loss of activity, but convert an initially homogeneous material into a mixture of different materials, some of which, although soluble, are even more easily sedimentable by high-speed centrifugation than the native antigen. The native antigen is a more powerful antigen and less toxic than AP. The greater antigenicity is associated with the state of greater aggregation ; the lesser toxicity with the presence of the PL1 or S fraction. The larger particles, being less readily eliminated, have time to stimulate the greater antibody response, and being less diffusible, have a less immediate toxic effect. There is a demonstrable relation between antibody stimulation and the amount of subcutaneous tissue exposed to the action of AP. Four-tenths of a mg. of AP given intradermally at one time in ten separate 0·04-mg. doses is more antigenic than 0·4 mg. given in one intradermal dose. This procedure presumably retards the elimination of the antigen. The consequent increase in antibody formation recalls Hartley's (1935) demonstration of the increase in antigenicity of intraperitoneally injected toxoid when given in a large volume. The large [PLAPS] particles appear to be eliminated even more slowly, providing an antigenic stimulus over a relatively long period. Intradermal injections of [PLAPS] and AP induce a skin sensitivity to subsequent injections of AP, and in a normal animal [PLAPS] remains in the injection site so long that a late allergic reaction occurs there as a result of general sensitivity induced by the dose itself.

The purity of our AP preparation cannot be established chemically, and it is impossible at present to exclude contamination with PL1 or S in amounts less than 5 per cent. The presence of small amounts of PL1 or S may determine the antigenicity of AP, but this seems unlikely, since any treatment known to remove or destroy PL1 or S may be prolonged without change in the antigenic power of AP. That agar derivatives are responsible to any extent for the carbohydrate content of our AP preparations is also unlikely, for purified AP from broth- or inspissated serum-grown bacteria have the same composition as AP from agar-grown bacteria.

The fact that the precipitin optima and end-points occur constantly in dilutions as high as 1 : 30,000 and 1 : 2,500,000 is not evidence that AP is a pure substance, but the end-point compares favourably with that of other purified bacterial SSS. All fractionations that did not destroy AP, including precipitation with antisera from dilute solutions, failed to increase the end-titre. Moreover, the chemical and serological properties of AP prepared from 15 separate harvests of the Y6 strain of *Br. melitensis* have proved remarkably constant.

There are several chemical properties which may be used to predict accurately the serological activity of a preparation of disaggregated antigen (AP) free from the lipoids PL1 and the protein-like fraction S. In the first place the preparation should be free from agar, as estimated by colorimetric tests, and from protein, as detected by the biuret or Millon reaction, or better still by the Sakaguchi reaction. It should, however, contain 14 per cent. of total carbohydrate and 5·4 per cent. of nitrogen. The most characteristic properties are the presence of the insoluble fraction called PL2 and the presence of the easily hydrolysable phosphate. No fraction has been isolated from any

batch of bacteria which has serological activity, and which does not contain an amount of these two components that is consistent with the serological activity found ; nor has any fraction which gives PL2 on hydrolysis been found that lacks serological activity.

Native antigen or [PLAPS] is found in the supernatant fluid of a " wash-off " of agar-grown bacteria, after it has stood in the cold for one week with 2 per cent. phenol. It must be studied when fresh and in solution. Different preparations of native antigen have consistent chemical, centrifugal and serological properties ; but the mixture of products of different degrees of disaggregation which results when old suspensions, or bacteria that have been dried or frozen, are used for the preparation, is not homogeneous when centrifuged, and gives a variable serological response, depending on the distribution of particle size. The nature of a preparation of this type is most easily defined by a study of the phospholipoid fraction from it. The trichloracetic acid extraction method of Lisbonne and Monnier (1936) and the tryptic digestion of acetone-dried bacteria employed by Pennell and Huddleson (1937) destroy a large part of the [PLAPS] complex, though [PLAPS]-like fractions may be obtained from these extracts by high-speed centrifugation. Antigen preparations which, like that of Pennell and Huddleson (1937), have been made from dried bacteria and have been precipitated with alcohol, will consist, in part at any rate, of the mixture of decomposition products mentioned above. Treatment with acetic acid disaggregates the [PLAPS] complex and produces AP. The soluble portion of the wash-off also contains agar derivatives, but these cannot be avoided by using triple-washed bacteria, for extracts of these contain large quantities of agar derivatives.

Pop *et al.* (1938) have prepared a solution of " complete antigen " which presumably contains material similar to our native antigen, but their method of preparation—extraction of the bacteria with trichloracetic acid—will leave a large part of the antigen in the bacterial residue, and the extract will contain substances other than the antigen. This is borne out by the relatively low precipitation end-point of 1 : 100,000. Their fraction and that of Lisbonne and Monnier (1936) produce in sensitized rabbits a reaction like our AP reactions. The absence of [PLAPS] from their preparation is further demonstrated by the absence of the characteristic late skin reaction in their control rabbits.

The instability of native antigen makes its definition difficult. Its capacity for forming oriented precipitates with antibodies suggests that the surface is not serologically homogeneous, but that some parts have greater combining power than others. Fresh preparations show anisotropy of flow. That is, the fluids are not birefringent when at rest, but become so when subjected to shear. The underlying theory of anisotropy of flow is still uncertain, but it can be safely assumed that fluids exhibiting the phenomenon generally contain birefringent particles which depart markedly from spherical shape, *e. g.* are rods or plates.

The bacterial residue after extraction of native antigen seems to be largely protein, containing 10–14 per cent. *N* and 1–2 per cent. carbohydrate. The composition of [PLAPS] is relatively constant, and differs consistently from that of the bacterial residue. This, and the constancy of the sedimentation rate, make it clear that we are not dealing with indiscriminate bacterial

fragments, nor, in view of the existence of [PLAPS] in large quantities in fresh phenolized bacterial suspensions, is it likely that the oriented nature of the aggregate is an artefact induced by chemical or physical treatment. This point cannot be proved conclusively. Morgan (1937) has noted a shimmer in many solutions of bacterial antigens extracted with diethyleneglycol. Here also, presumably, the constituent particles are large and anisodimensional.

The position of [PLAPS] in the bacterial cell is still uncertain. But the study of S⟶R variation in *Br. melitensis*, and of the associated changes in agglutination reactions, indicates that the progressive loss of smooth antigen is accompanied by a gradual uncovering of an R antigenic surface. We suggest that [PLAPS] is predominant in the surface of the bacterium in the form of a shell or capsule, in which PLAPS units are built in such a way that the shell of [PLAPS] disrupts into plate or rod-like pieces. The yield of [PLAPS] from *Br. melitensis*, a cocco-bacillus 0·6 × 1·6μ, has never exceeded 10 per cent. of the dry weight of the organism. If we assume that the bacterial mass and [PLAPS] are hydrated to the same extent, it is easy to calculate that a surface layer of [PLAPS] one-tenth of the bacterial weight would be 10–20 mμ. thick. The errors in any of these assumptions have but little effect on this figure. It is clear that a capsule of this thickness would not be detectable microscopically. This is not evidence that [PLAPS] is in the form of a capsule, but that if it were so, it would not be visible.

The A and M antigens postulated by Wilson and Miles (1932) have not been isolated. This failure may be interpreted in two ways: Firstly, the two antigenic determinants may be carried on the molecule of AP; secondly, molecules with different antigenic determinants may resemble one another too closely to be separable by the methods we have used. The serological distinction of A from M antigens on the surface of the whole bacterium depends on the monospecific agglutinating serum. By analogy, the distinction of A and M antigens in SSS solutions should be demonstrable by monospecific precipitating sera. Absorption of precipitating sera yields solutions that precipitate SSS monospecifically, but these do not necessarily contain only monospecific antibodies. The precipitating power of an antibody depends not only on the presence of appropriate receptors, but on a certain degree of " ripeness ", and the absorption may have left non-precipitating heterospecific antibodies that are capable of modifying the reactions of truly monospecific precipitating antibodies. Moreover, our experience with *Brucella* precipitating systems confirms the suggestion put forward by Miles (1939) that the *Brucella* antibody molecules may be anti-A, anti-M, or anti-AM (*i. e.* carrying both kinds of receptors). If anti-AM antibodies predominate, differential absorption is difficult. The technique of precipitin absorption is full of pitfalls, not the least of which is the presence of small quantities of soluble antigen-antibody complexes capable of modifying the reaction of the free antibody. Even with careful quantitative control of precipitin tests the serological distinction of A and M antigens is open to question. *Brucella* fractions and the antibodies in *Brucella* antisera are complex, and the mechanisms of precipitin and agglutination reactions postulated for simpler systems with predetermined antigens will not necessarily hold. This is well shown in the determinations of nitrogen precipitated from *Brucella* antisera by endoantigens, recorded by Pennell and Huddleson (1938).

The homologous systems yield results comparable with those found for homogeneous solutions of single antigens by Heidelberger and Kendall (for refs. see Marrack, 1938), but the heterologous systems submit to no such simple analysis.

A more dependable definition of the A- or M-quality of fractions has been obtained by measuring their capacity to inhibit monospecific agglutinating systems. The M content of our best AP fractions is 80 times that of the A content, and *Br. abortus* SSS contains small quantities of M and a large amount of A. In the gross, the SSS from the two species are serologically distinct.

Native antigen apparently contains R antigen in addition to A and M, but the lack of homogeneity of all but fresh preparations makes quantitative absorption and inhibition tests difficult. The matter is still under investigation. No fraction from the most gently treated growths of fully smooth bacteria contained any other than the serologically recognizable A and M antigens—a finding in agreement with that of Priestley (1938), who failed to demonstrate any evidence of a labile antigenic component in the closely related *Br. abortus*.

The fractionations of *Br. abortus* we have so far carried out have yielded [PLAPS]-like and AP-like substances. *Br. abortus*-AP of high optimum or end-titre is relatively difficult to obtain, and reacts more slowly with homologous antibody than does *Br. melitensis*-AP. *Br. melitensis*-AP is mostly M antigen, but there is no reason to suppose that its "A-activity" is necessarily carried by a particle distinct from that carrying the M. The same reasoning applies to *Br. abortus* extracts. Moreover, the specificity of the A antigen may depend on the integrity of structures more complex than AP.

The results of our chemical and serological survey are consistent with Wilson and Miles's hypothesis that the antigens of smooth *Brucella* strains contain an M and an A antigen. But it must be emphasized that this hypothesis still depends fundamentally on the validity of these authors' interpretation of the monospecific agglutinating serum, and it cannot be regarded as established until two distinct antigenic substances are isolated, each free from contamination with the other.

SUMMARY TO THE THREE PAPERS IN THIS SERIES ON THE ANTIGEN OF *BRUCELLA MELITENSIS*.

Treatment of suspensions of smooth *Br. melitensis* with chloroform-water, phenol, etc., brings into solution a substance which can be sedimented at 14,000 r.p.m. and which appears to contain the antigenic components of the whole organism. This substance is unstable towards organic solvents and towards drying, and gives viscous opalescent solutions that show anisotropy of flow. A mixture of lipoids can be separated from it by treatment with organic solvents, and a protein-like material by treatment with acetic acid. Agents such as ammonium sulphate, acetic acid and sodium dodecyl sulphate affect the particle size of the resulting substance; its molecular weight can vary between 100,000 and 1,000,000.

Precipitates of native antigen with specific antisera are not readily soluble in excess of antigen. With some rabbit antisera the suspension of antigen-antibody precipitate are orientatable and the individual aggregates show a characteristic microscopic structure. The degraded antigen gives

a precipitate, soluble in an excess of antigen, similar to those given by most bacterial antigens ; it contains the A and M antigens only, whereas the native antigen apparently contains come rough antigen as well. Preparations in different states of aggregation differ in antigenicity, toxicity, immunizing power, and in the reactions given when they are injected into the skins of specifically sensitized rabbits. Native antigen is more antigenic and less toxic than degraded antigen and it gives skin-lesions with maxima after 4 and after 10 days, whereas degraded antigen gives the first maximum only.

On gentle acid hydrolysis the degraded antigen is split, giving the formyl derivative of an amino-polyhydroxy compound and an insoluble phospholipoid. The former is an inhibitory hapten, but it loses this power, and also a phosphate group, on further hydrolysis ; it is neither toxic nor antigenic.

The chemical and physical properties of the antigen in its different states of aggregation are described in detail, as are the processes used for the removal of agar and of contaminants of bacterial origin. The properties of the substances split off from purified native antigen by the various treatments to which it has been subjected are summarized.

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SURVEY OF PAPERS.

In the second paper dealing with the action of proteolytic enzyme on antitoxic sera POPE describes the results obtained after a limited amount of enzyme action on antitoxins followed by critical heat denaturation of non-antitoxic protein. Several enzymes possess the property of altering the antitoxic pseudoglobulin and converting it into two protein fractions, one of which is non-antitoxic and easily denatured in acid solutions at high temperatures. The antitoxin is not destroyed by this treatment (p. 201).

POPE and HEALEY have used diphtheria antitoxin which has already been purified by enzyme methods as the starting material in an attempt to secure pure antitoxin. After specific precipitation of the antitoxin by toxin, the toxin in the precipitate was destroyed with pepsin and the liberated antitoxin purified. The product obtained was completely precipitable with toxin, and appeared to be free from other antibodies to *C. diphtheriæ* (p. 213).

In a previous paper DELAFIELD and SMITH recorded that toxic fractions derived from *Bact. ærtrycke* by a process involving tryptic digestion increased the oxygen uptake of brain suspensions in glucose and inhibited the oxygen uptake of muscle suspensions in succinate. It was then believed that these effects might have some bearing on the problem of the toxic action of the organisms *in vivo*. It has now been shown that this is wrong in that these effects were not produced by the bacterial cell, but by some material present in the trypsin preparation used. This material is also present in the pancreas. The influence of the pancreas in producing these modifications of the oxygen uptake of brain and of muscle suspension *in vitro* may be of physiological importance (p. 216).

Mottram and Doniach have shown that cultures of *Paramœcium caudatum* which have been treated with various carcinogenic agents are killed by near-ultra-violet light. DONIACH describes a method of measuring this photodynamic sensitivity of paramœcia, and has found that it is greatly increased by contact with the carcinogenic agents in the dark. Several non-carcinogenic light-sensitizers are much less active in this respect. The photo-oxidation products of 3–4 : benzpyrene are strongly active, and it is suggested that the photodynamic and tumour-producing properties of the carcinogenic hydrocarbons may both be due to a process of oxidation (p. 227).

It has always been assumed in plague prophylaxis that a highly virulent smooth organism was necessary for the preparation of the vaccine. SCHÜTZE, using rats and mice, found no correlation between the prophylactic efficiency of a vaccine and the virulence and colonial "smoothness" of the culture from which it was obtained. On the other hand, the amount of "envelope" antigen was highly important in the rat, but much less so in the mouse (p. 235).

ABDALLA and MCLEOD prepared a stable form of streptococcal "serum-free" hæmolytic filtrate by using as medium an extract of meat prepared at a temperature below 75°. They confirmed the work of Todd in finding that such filtrate was actively antigenic and lethal for the rabbit; they differed from him in finding that filtrates containing horse-serum did not produce antibody against "serum-free" hæmolysin, although these filtrates immunized rabbits against a lethal dose of "serum-free" hæmolysin. Rabbit serum hæmolytic filtrates produced both antilytic and protective antibodies against "serum-free" hæmolysin (p. 245).

MACCALLUM, FINDLAY and SCOTT describe a new virus. Two strains have been isolated in mice injected with the cerebro-spinal fluid of two patients with symptoms of benign aseptic lymphocytic meningitis. The disease in mice is very similar to that produced by the virus of lymphocytic choriomeningitis, but there is no apparent relation between the two viruses. The question whether the virus is of human or murine origin requires further observation (p. 260).

ANDERSON and MACKIE have confirmed again the hæmolytic effect of colloidal silicic acid and tannic acid (p. 270).

MILES and PIRIE continue the description of the antigen from *Br. melitensis*. The third part of the study is concerned with preliminary results of hydrolysis, and with the biological properties of the antigen and of its breakdown products. The simplest material that will still precipitate with antisera at high dilution is a complex of a phospholipoid and the N-formyl derivative of an amino polyhydroxy substance; this complex also contains a small amount of phosphate readily removable by hydrolysis. The toxicity of the native antigen is less, and the antigenicity greater, than that of the partly broken-down materials.

A discussion of the results in all three papers of this series is included in the paper (p. 278).